AD_____

GRANT NUMBER: DAMD17-94-J-4447

TITLE: Plasmin-Cellular Interaction in Breast Cancer Invasion and Metastasis

PRINCIPAL INVESTIGATOR: Steven L. Gonias, M.D.

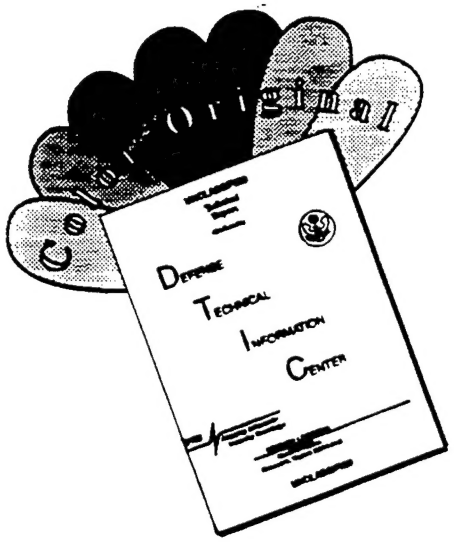
CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22906

REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF COLOR PAGES WHICH DO NOT REPRODUCE LEGIBLY ON BLACK AND WHITE MICROFICHE.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (30 Sep 95 - 29 Sep 96)

4. TITLE AND SUBTITLE
Plasmin-Cellular Interaction in Breast Cancer Invasion and Metastasis

5. FUNDING NUMBERS
DAMD17-94-J-4447

6. AUTHOR(S)
Steven L. Gonias, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, Virginia 22906

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Invasion and metastasis of breast cancer cells requires the function of a cell-surface proteinase cascade in which the proteinase, plasmin, plays a prominent role. Studies of a variety of cell types have shown that the tumor invasion proteinase cascade functions optimally only when plasmin is bound to the cell surface. Different macromolecules may be responsible for plasmin-binding in different cell types. The goal of this research program has been to identify macromolecules responsible for cell-surface immobilization of plasmin in breast cancer cells. In the second year of this research program, we have made significant progress and completed studies identifying cytokeratin-8 as the major plasminogen receptor in three different breast cancer cell lines. Furthermore, we have demonstrated the ability of cytokeratin-8, which is secreted by breast cancer cells, to promote the activation of proteinases involved in tumor invasion in the pericellular spaces. These studies have been published in major journals. We are now exploring the role of cell-surface cytokeratin-8 in breast cancer cell adhesion, migration, and invasion. We have prepared a mutated form of the cytokeratin-8 cDNA for transfection (transfer of function) experiments. Finally, we have initiated studies on plasminogen activator binding to breast cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Invasion, proteinases, Plasmin, Urokinase, cellular receptors	38
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

SD Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

SD For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

SD In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

SD In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

S/D 10/24/96

PI - Signature Date

Steven L. Gonias
DAMD 17-94-J4447

Table of Contents:

Steven L. Gonias
DAMD 17-94-J4447

Introduction:

Cancer invasion and metastasis are promoted by proteinases which are organized on the cell surface by a series of cellular receptors. These proteinases form a cascade which culminates in the digestion of basement membrane and extracellular matrix proteins, allowing tumor cells to penetrate through tissue (1). At the top of the tumor invasion cascade, is urokinase-plasminogen activator (u-PA), a plasminogen activator which binds to a specific cellular receptor called uPAR (2,3). In breast cancer, cellular expression of u-PA has been correlated with an aggressive, malignant phenotype (4,5).

When u-PA is bound to uPAR, the proteinase effectively activates cell-associated plasminogen to form plasmin. Plasmin, in turn, digests glycoprotein components of the extracellular matrix and may activate other proteinases (6,7). A second plasminogen activator, tissue type plasminogen activator (t-PA), also binds to tumor-cell surfaces and may substitute for u-PA in certain circumstances (8).

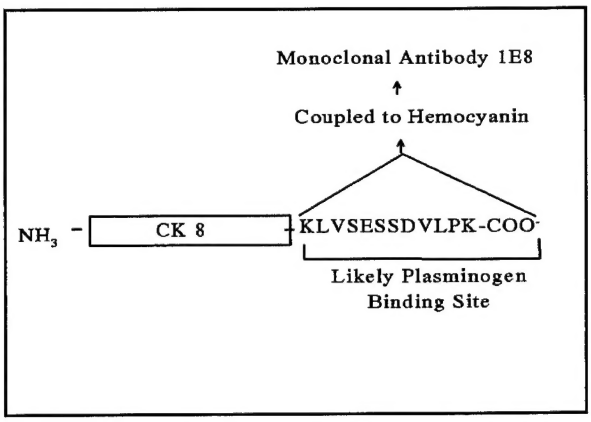
Migrating cells show the capacity to polarize the tumor invasion proteinase cascade to the lead edge of migration (1). Thus, the cell may selectively digest structural proteins in the direction of migration. When we began our work, the nature of the cellular binding site for plasminogen in breast cancer cells was unknown. Plasminogen binding sites have been described in other cell types, but in most cases, subsequent studies demonstrated that these binding sites are not responsible for a substantial fraction of the plasminogen binding capacity (9-12). By contrast, a growing body of literature supports the hypothesis that plasminogen binding to the cell surface is ESSENTIAL for the optimal function of the tumor invasion proteinase cascade (13).

The major goal of this research program has been to identify and characterize plasminogen binding sites in breast cancer cells. We consider this binding site an exciting and unexplored target for potential disruption of the tumor invasion proteinase cascade. The body of literature, outlined above, supported the selection of this interaction for investigation.

Body:

In my first progress report, I described our studies which allowed us to identify cytokeratin 8 as a plasminogen-binding protein in the plasma membranes of hepatocytes and hepatocellular carcinoma cells. A big step forward in this original work was our discovery that the plasminogen binding site was detergent-insoluble. Of the many detergents studied, only SDS solubilized the major plasminogen-binding component in the plasma membranes and we were able to determine through a series of biochemical studies that the binding protein was cytokeratin 8.

Cytokeratin 8 is an intermediate filament protein and a major component of the cytoskeleton of numerous simple epithelial cells and epithelial cell-derived neoplasms. However, it was not clear whether any cytokeratin 8 was present on external cells surfaces. We utilized immunofluorescence microscopy and immunoelectron microscopy to definitively demonstrate that cytokeratin 8 is present on the external surfaces of liver cancer and breast cancer cell lines. This work was published in *J. Cell Sci.* and a copy of the manuscript is included in the Appendix.



The second major phase of our research has included studies which have identified cytokeratin 8 as a true receptor for plasminogen in breast cancer cells. The early stages of this work were described in my first progress report. Substantial follow-up has culminated in the publication of a paper in *J. Biol. Chem.* (submitted in revised form July 18, 1996), a copy of which is included in the Appendix.

I will now summarize the major findings presented in the published manuscript. References to figures will be to those appearing in the *J. Biol. Chem.* paper, as numbered in the text of that manuscript. Initially, we performed "plasminogen overlay" or "ligand blot" experiments to characterize plasminogen-binding components in the plasma membrane fractions of three different breast cancer cell lines. Although these studies identified candidate receptors, one must be cautious in interpreting the results since plasma membrane components with plasminogen-binding capacity may or may not be oriented in a manner so that the critical plasminogen-binding domain is on the external surface of the cell and available to ligand. Figure 3 in the manuscript shows that each of the three breast cancer cell lines contained a major plasminogen-binding component with an apparent mass of about 55-kDa in the plasma membrane fraction. Western Blot analysis of the plasma membrane preparations confirmed that the 55-kDa protein is cytokeratin 8.

To demonstrate whether cytokeratin 8 is an important plasminogen receptor in intact breast cancer cells, we raised a very important reagent, monoclonal antibody 1E8. Previous studies suggested that the plasminogen-binding site in cytokeratin 8 included the C-terminal lysine residue and potentially a second lysine residue. Thus, the synthetic peptide shown in the accompanying figure (on this page) was coupled to hemocyanin via an N-terminal Cysteine residue and used as an immunogen to generate antibody 1E8.

Monoclonal antibody 1E8 recognized cell-surface cytokeratin 8 in nonpermeabilized breast cancer cells, as determined by immunofluorescence microscopy (Figure 2). Furthermore,

radioiodinated 1E8 bound in a specific and saturable fashion to the surfaces of MCF-7 breast cancer cells (Figure 5). Scatchard transformation of the ^{125}I-1E8 binding data indicated that there were approximately $1.5 \pm 0.5 \times 10^6$ binding sites per cell. This was an important result since it suggested that the number of exposed 1E8 epitopes was nearly the same as the number of plasminogen binding sites on the surfaces of the MCF-7 cells.

A series of competition experiments was then performed to generate the most important results of the study. In these experiments, we demonstrated that monoclonal antibody 1E8 displaces plasminogen from key binding sites on the surfaces of breast cancer cells. However, the experiments were not completely straightforward since some immunoglobulin Fc domains demonstrate affinity for plasminogen. Thus, we prepared Fab fragments of 1E8 and then performed our competition experiments under a variety of conditions to be absolutely certain that 1E8 inhibited plasminogen binding to cell surfaces by competition at the plasma membrane and not by a solution-phase interaction. The results, presented in Figure 6, conclusively demonstrated substantial competition between 1E8 Fab and plasminogen for cell surface binding in MCF-7 cells and in other breast cancer cell lines. By comparison, a fibroblast cell line, which does not express cytokeratin 8, was unaffected by 1E8 Fab. Most importantly, as shown in Figure 8, monoclonal antibody 1E8 significantly inhibited the greatly accelerated rate of plasminogen activation which occurs on breast cancer cell surfaces. Thus, in 1E8, we developed a functional reagent capable of inhibiting the cell-surface tumor invasion proteinase cascade. A disclosure has been filed regarding this antibody and the Department of the Army has been notified through the proper channels.

Cytokeratin 8 released by breast cancer cells - In addition to cell-associated cytokeratin 8, we have become increasingly interested in cytokeratin 8 that is secreted by breast cancer cells. There are two reasons for this. First, we initially developed the hypothesis that cell surface cytokeratin 8 may first be secreted by cells and then bind tightly to the plasma membrane, thereby functioning as an "exogenous" membrane receptor. Some new studies, which I will not review here, seem to disprove this hypothesis; however, this remains an important topic of investigation for us.

The second reason why we are interested in secreted cytokeratin 8 reflects the possibility that this protein could promote the activation of proteinases in the pericellular spaces and thus modify the extracellular matrix, facilitating penetration of tumor cells. To address this question, we performed experiments to determine whether cytokeratin 8 released by breast cancer cells was competent to bind plasminogen and/or promote plasminogen activation. I will review these results, referring to the figures that are presented in the accompanying manuscript published in the *Biochem. J.* As shown in Figure 1, we initially demonstrated that cytokeratin 8 is present in the conditioned-medium of breast cancer cells and that the secreted cytokeratin 8 was competent to bind plasminogen. This implied that the C-terminus was intact since we understand the importance of this region of cytokeratin 8 in plasminogen-binding interactions. By molecular exclusion chromatography, we determined that the secreted cytokeratin 8 was in the form of variably sized polymers (Figure 2).

Most importantly, we determined that the secreted cytokeratin 8 polymers enhance the activation of plasminogen by t-PA. Based on this observation, we conducted a more detailed analysis of the mechanism by which cytokeratin 8 promotes plasminogen activation. The experiments are presented in the remaining figures of the manuscript. We propose that cytokeratin 8 promotes plasminogen activation by forming a ternary complex with plasminogen and t-PA. In this ternary complex, the K_m for the activation of plasminogen by t-PA is significantly decreased, mimicking the effects of fibrin on plasminogen activation. As would be predicted by our model, the activation of plasminogen by u-PAwas not promoted by solution-phase cytokeratin 8. We predict that this will not be the case when we analyze the activation of plasminogen by u-PA on intact breast cancer cells. Such studies are planned for the upcoming year.

New Directions - With these first three manuscripts, we have made substantial progress towards completing three of our original five specific aims. I will now outline new directions which are underway in this laboratory. The most important focus for us is to determine the role of cell-surface cytokeratin 8 in breast cancer cell invasion. To accomplish this goal, we have revised our original plans and initiated studies with two new cell lines, MDA MB9355 and MDA MB231. By contrast with the lines which we have been studying to date, MCF-7 and BT20, these new cell lines are highly invasive in both in vitro tumor invasion model systems and in immune-compromised (nude) mice. Before the four years of this project are completed, we would like to complete all of the proposed tumor invasion experiments in the in vitro model systems proposed in the original grant application and also translate our results into the nude mouse system. In initial studies, we have characterized plasminogen binding to the new cell lines. The binding capacity is over 1×10^6 sites per cell with both cell lines. In preliminary Matrigel invasion assays. MDA MB231 cells show about twice the invasive capacity of MDA MB9355 cells and both cell lines are substantially more invasive than BT20. However, the level of cytokeratin 8 expression by the two new cell lines is lower than BT20 and dependent on cell density. Thus, it will be extremely important for us to characterize cell-surface expression of cytokeratin 8 in relation to total cellular expression. We have no reason to believe that these two properties will be related.

As an important control experiment, we have examined a melanoma cell line, DM14, which expresses cytokeratin 8 aberrantly. Although these cells have substantial amounts of cytokeratin 8, there is no cell-surface cytokeratin 8. This is consistent with our present model in which we hypothesize that expression of cell-surface cytokeratin 8 is dependent on desmosomes or hemidesmosomes, which are unique to epithelial cells.

Our new model has a direct impact on our "transfer of function" experiments, which were described in highly preliminary form in my first progress report. We have available in the laboratory a full-length cytokeratin 8 cDNA expression construct together with a cytokeratin 18 expression construct. We have been successful in transferring these cDNAs into fibroblast cell lines and the resulting constructs have caused the expression of cytokeratin 8 in the transfected cells, as

determined by Western Blot analysis. However, the transfected cells have not expressed cell-surface cytokeratin 8 as determined by immunofluorescence microscopy or monoclonal antibody ^{125}I-1E8 binding experiments. We think that this may be due to the lack of desmosomes and/or hemidesmosomes in these cell types. Alternatively, the expression of cell-surface cytokeratin 8 may be dependent on the level of total cellular expression. This is an important point of future investigation. To address this issue, we have mutated the cytokeratin 8 cDNA, replacing the C-terminal lysine with a glutamine. This construct should allow for the expression of a mutated cytokeratin 8 which polymerizes normally but lacks plasminogen binding capacity. We are excited regarding the potential use of this construct, since it may represent a dominant negative form of cytokeratin 8, in regard to plasminogen binding, for future studies. A dominant negative cytokeratin 8 could be transfected into breast cancer cells and potentially decrease the plasminogen binding capacity of these cells.

A final focus of current investigation involves the binding site for t-PA in cytokeratin 8, following up on the manuscript published in the *Biochem. J.* We have demonstrated that t-PA binds specifically and saturably to MCF-7 cells. When these cells are extracted with a variety of detergents, including Triton X-100, CHAPS, or sodium deoxycholate, the t-PA remains bound to the detergent-insoluble fraction. These results suggest that the t-PA is associated with cytokeratin 8 in the intact cells, or with another protein that may be affiliated with intermediate filaments.

Literature cited in report:

1. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W. (1991) *Cell* **64**, 327-336

2. Roldan, A.L., Cubellis, M.V., Masucci, M.T., Behrendt, N., Lund, L.R., Dano, K., Apella, E., and Blasi, F. (1990) *EMBO* **9**, 467-474

3. Behrendt, N., Ronne, E., Ploug, M., Petri, Tl, Lober, D., Nielsen, L.S., Schleuning, W.-D., Blasi, F., Apella, E., and Dano, K. (1990) *J. Biol. Chem.* **265**, 6453-6460

4. Duffy, M.J., Reilly, D., O'Sullivan, C., O'Higgens, N., Fennelly, J.J., and Andreasen, P. (1990) *Cancer Res.* **50**, 6827-6829

5. Bianchi, E., Cohen, R.L., Thor, A.T., Todd, R.L., Mizukami, I.F., Lawrence, D.A., Ljung, B.M., Shuman, M.A., and Smith, H.S. (1994) *Cancer Res.* **54**, 861-866

6. Liotta, LA., Goldfarb, R.H., Brundage, R., Siegal, G.P., Teranova, V., and Gabrisa, S. (1981) *Cancer Res.* **41**, 4629-4635

7. Mignatti, P., Robbins, E., and Rifkin, D.B. (1986) *Cell* **47**, 487-498

8. Alizadeh, H., Ma, D., Berman, M., Bellingham, D., Comerford, S.A., Gething, M.J.H., Sambrook, J.F., and Niederkorn, J.Y. (1995) *Curr. Eye Res.* **14**, 449-458

9. Miles, LA., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K., and Plow, E.F. (1991) *Biochemistry* **30**, 1682-1691

10. Hajjar, K.A., Jacovina, A.T., and Chacko, J. (1994) *J. Biol. Chem.* **269**, 21191-21197

11. Cesarman, G.M., Guevera, C.A., and Hajjar, K.A. (1994) *J. Biol. Chem.* **269**, 21298-21203

12. Redlizt, A., Fowler, B.J., Plow, E.F., and Miles, L.A. (1995) *Eur. J. Biochem.* **227**, 407-415

13. Kobayashi, H., Shinohara, H., Takeuchi, K.I., Itoh, M., Fujie, M., Saitoh, M., and Terao, T. (1994) *Cancer Res.* **54**, 844-849

Conclusions:

The following is a list of the major conclusions drawn from the work conducted during the first two years of the research program.

1) Cytokeratin 8 is a major plasminogen receptor in breast cancer cell lines, accounting for as much as 80% of the total plasminogen binding capacity of these cells.

2) Cytokeratin 8 is critical for the enhanced activation of plasminogen by plasminogen activator on the surfaces of breast cancer cells.

3) Monoclonal antibody 1E8, generated against the C-terminus of cytokeratin 8, can decrease plasminogen binding and plasminogen activation by breast cancer cells.

4) Cytokeratin 8 which is secreted by breast cancer cells may promote the activation of plasminogen in the pericellular spaces. The mechanism probably involves the formation of a ternary complex in which the K_m for plasminogen activation is decreased.

In the body of this progress report, I have attempted to outline the points of greatest emphasis for our continued work. We remain basically aligned with our original objectives, outlined in the original five specific aims. One deviation is the enhanced use of molecular genetic techniques to tackle the major problems involved in cytokeratin 8 as a promoter of the cell-surface tumor invasion cascade. Although our studies indicate a prominent role for cytokeratin 8, we will evaluate other potential plasminogen binding sites if our results indicate that such sites may be important, as

outlined in the original grant application. Furthermore, we are still committed to an analysis of breast cancer cells derived from surgically-excised tumor specimens, in the final two years, as indicated in Specific Aim 3.

References:

The following is a complete list of references resulting from the first two years of grant support. The first of three references was reported in the first progress report. The subsequent two references are reported here.

1. Hembrough, T.A., Vasudevan, J., Allietta, M.M., Glass, W. F., II, and Gonias, S.L. A cytokeratin 8-like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines. *J. Cell Science* 108:1071-1082, 1995.

2. Hembrough, T.A., Li, L., and Gonias, S.L. Cell-surface cytokeratin 8 is the major plasminogen receptor on breast cancer cells and is required for the accelerated activation of cell-associated plasminogen by tissue-type plasminogen activator. *J. Biol. Chem.* 271:25684-25691, 1996.

3. Hembrough, T.A., Kralovich, K.R., Li, L., and Gonias, S.L. Cytokeratin 8 released by breast carcinoma cells in vitro binds plasminogen and tissue-type plasminogen activator and promotes plasminogen activation. *Biochem. J.* 317:763-769, 1996.

List of Personnel Receiving Pay from this Research Grant
Steven L. Gonias, Principal Investigator
Todd Hembrough, Graduate Research Associate
Kristen Kralovich, Graduate Research Associate
Sari Benmeir, Research Technologist
Megan McCabe, Research Technologist
Janice Wen, Research Technologist

Journal of Cell Science 108, 1071-1082 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

A cytokeratin 8-like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines

Todd A. Hembrough, Jayanand Vasudevan, Margaretta M. Allietta, William F. Glass II and Steven L. Gonias*

Departments of Pathology and Biochemistry, University of Virginia Health Sciences Center, Box 214, Charlottesville, VA 22908, USA

*Author for correspondence

SUMMARY

Plasminogen binding to cell surfaces may be important for tumor invasion and other processes that involve cellular migration. In this investigation, the principal plasminogen-binding protein was identified in the plasma membrane fraction of rat hepatocytes. The protein had an apparent mass of 59 kDa, was insoluble in a spectrum of detergents, and was identical to cytokeratin 8 (CK 8) as determined by sequence analysis of nine amino acids at the N terminus of two cyanogen bromide fragments. The 59 kDa protein bound CK 8-specific antibody in western blot analyses. These studies demonstrate that CK 8 or a CK 8-like protein binds plasminogen. Given this newly determined and potentially important CK 8 function, immunofluorescence and immunoelectron microscopy studies were performed to determine whether CK 8 may be present on the external surfaces of unpermeabilized, viable hepatocytes. All of the cells in each preparation were immunopositive with two separate CK 8-specific antibodies. A punctate pattern of immunofluorescence was detected on the cell surface with approximately even intensity from cell to cell. By immunoelectron microscopy, CK 8 was preferentially associated with microvilli. In order to determine whether other epithelial cells express cell-surface CK 8, immunofluorescence and immunoelectron microscopy studies were performed with HepG2 hepatocellular carcinoma cells and with BT20 and MCF-7 breast carcinoma cells. The pattern of antigen expression was equivalent with each cell type and comparable to that observed with hepatocytes. These studies support the hypothesis that CK 8 is associated with the external cell surface where it may express important proteinase receptor function.

Key words: plasminogen, cytokeratin, plasma membrane, intermediate filament

INTRODUCTION

Plasmin is a serine proteinase that plays a central role in fibrinolysis. At the cell surface and in the pericellular spaces, plasmin cleaves many substrates other than fibrin, including: glycoprotein components of the extracellular matrix (Liotta et al., 1981), zymogen forms of certain matrix metalloproteinases (He et al., 1989), glycoprotein IIIa (Pasche et al., 1994), growth factor precursors such as latent transforming growth factor-β (TGF-β) (Lyons et al., 1988), insulin-like growth factor-binding protein (Campbell et al., 1992), interferon-γ (Gonias et al., 1989b), the thrombin receptor (Turner et al., 1994), and the type III TGF-β receptor/betaglycan (LaMarre et al., 1994). These enzyme-substrate reactions suggest multiple mechanisms by which plasmin may regulate cellular growth, differentiation and cellular migration.

The diverse continuum of plasmin substrates emphasizes the importance of regulating this proteinase within the pericellular spaces. Plasmin regulation is accomplished at three levels. The first is activation, which involves the proteolytic conversion of single-chain plasminogen into the active two-chain structure (Ponting et al., 1992). Once activated, plasmin is regulated by proteinase inhibitors, including α_2-antiplasmin and α_2-macroglobulin (Ellis and Dano, 1992; Gonias, 1992; Vassalli et al., 1991). Plasmin is also regulated spatially by noncovalent binding interactions that localize and concentrate the proteinase (and zymogen) in specific microenvironments. Reversible plasmin(ogen) binding to cell surfaces, fibrin clots and specific extracellular matrix proteins is mediated by five homologous plasmin(ogen) kringle domains (Ponting et al., 1992). The kringle domains have binding sites with affinity for lysine residues in other proteins. Association of plasminogen with many proteins depends on the relatively high-affinity interaction of the kringle-1 (K1) domain with a carboxy-terminal lysine residue (Christensen, 1985; Fleury and Anglés-Cano, 1991; Miles et al., 1991).

Plasmin(ogen)-binding proteins, including those present on cell surfaces, not only localize plasmin near potential substrates, but also significantly alter the kinetics of plasminogen activation and plasmin inhibition (Ellis and Dano, 1992;

Gonias, 1992). Therefore, understanding the spectrum of physiologically significant plasminogen-binding proteins is important. Nucleated mammalian cells, including endothelial cells (Hajjar et al., 1986), monocytes/macrophages (Plow et al., 1986), hepatocytes (Gonias et al., 1989a) and tumor cells (Burtin and Fondaneche, 1988; Correc et al., 1990; Hall et al., 1990) express specific plasmin(ogen)-binding sites. The binding affinity is typically low (K_d~1.0 μM); however, the capacity is high (10^5-10^7 sites per cell). Specific plasminogen binding to all cell types can be completely inhibited by lysine analogues such as ε-amino caproic acid (EACA) (Hajjar et al., 1986; Gonias et al., 1989a), confirming the kringle domain-dependency of these interactions.

Plasminogen-cellular interactions may be mediated by different cellular proteins. α-Enolase was identified as a candidate plasminogen receptor in U937 monocytes (Miles et al., 1991). Although α-enolase is primarily an intracellular protein, FACS analysis was used to demonstrate that this antigen is present on cell surfaces. Annexin-2 is another primarily intracellular protein that may be found in association with the external surfaces of endothelial cells where it functions as a receptor for plasminogen and tissue-type plasminogen activator (Hajjar et al., 1994). Amphoterin is a candidate plasminogen receptor in developing brain (Parkkinen and Rauvala, 1991). It has also been shown that purified Heymann Nephritis autoantigen (gp330) binds plasminogen (Kanalas and Makker, 1991).

The present study was undertaken to identify major plasminogen binding proteins in hepatocytes. Similar analyses of epithelial cells have not been previously reported. Our results demonstrate that cytokeratin 8 (CK 8) or a CK 8-like protein is the major plasminogen binding protein in the plasma membrane fraction of rat hepatocytes. CK 8 is a basic-neutral intermediate filament protein, unique amongst cytokeratins in that it has a C-terminal lysine residue (Morita et al., 1988). CK 8 extends from the nucleus to the plasma membrane where it has extensive interactions with the internal leaflet and with various membrane-associated structures, including desmosomes and hemidesmosomes (Garrod, 1993; Owaribe et al., 1991). Epithelial cells, and especially carcinoma cells, secrete CK 8 or CK 8 fragments in vivo; these fragments are a major component of the long-recognized body-fluid complex, Tissue Polypeptide Antigen (TPA) (Leube et al., 1986; Weber et al., 1984). It has been proposed that CK 8 is also present on the external surfaces of epithelial cells (Godfroid et al., 1991; Donald et al., 1991). At least three mechanisms might hypothetically account for this. First, the CK 8 may penetrate through the plasma membrane as part of a multiprotein complex, despite the lack of a transmembrane domain. Second, CK 8 that is secreted by cells may tightly associate with the external surface of the plasma membrane (Riopel et al., 1993). Finally, since CK 8 covalently binds to plasma membrane lipids (Asch et al., 1993), it is conceivable that these complexes are translocated to the outer membrane leaflet.

The previous studies demonstrating cell-surface CK 8 have been contested on the basis that the experimental techniques utilized might detect primarily damaged or permeabilized cells (Riopel et al., 1993). Given the new function of CK 8 demonstrated here, we considered it important to re-evaluate the question of CK 8 exposure on cell surfaces. Therefore, in the second part of this study, immunofluorescence microscopy and immunoelectron microscopy experiments were performed. These studies detected, for the first time, CK 8 or a CK 8-like protein on the external surfaces of rat hepatocytes and HepG2 hepatocellular carcinoma cells. We also present a series of studies confirming the earlier, contested work regarding the presence of CK 8 on the external surfaces of breast carcinoma cells.

MATERIALS AND METHODS

Materials

$Na^{125}I$ was from Amersham International (Arlington Heights, IL). Aprotinin, chloramine T, EACA, phenylmethyl-sulfonyl fluoride (PMSF), bovine serum albumin (BSA), sodium deoxycholate and fetal bovine serum (FBS) were purchased from Sigma (St Louis, MO). Leupeptin, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), and collagenase D were purchased from Boehringer Mannheim. Williams E medium and Earle's balanced salt solution (EBSS) were from Gibco Laboratories (St. Lawrence, MA). D-Val-L-Leu-L-Lys *p*-nitroanilide (S-2251) was from Kabi Vitrum (Stockholm, Sweden).

Murine monoclonal antibody PCK-26, which recognizes CK 5, 6, and 8, and murine monoclonal antibody M20, which is specific for CK 8, were purchased from Sigma. The hybridoma, UCD/AB 6.01, was obtained from ATCC and expanded in ascites using $IRCF_1$ mice (Hilltop Labs, Scottdale, PA). AB 6.01 was purified from the ascites by Protein A-Sepharose chromatography. This antibody is specific for CK 8 but cross-reacts slightly with CK 18 (Chan et al., 1985). In our western blot analyses of purified rat hepatocyte CK, performed as previously described (Wolf et al., 1992), only CK 8 was detected by AB 6.01 (results not shown). A monoclonal IgG_1 specific for GTPase activating protein (p120-GAP) was kindly provided as a gift by Dr Sarah Parsons (University of Virginia).

Cell culture

All cell lines were obtained from ATCC. The human hepatocellular carcinoma cell line, HepG2 (Aden et al., 1979), was cultured in MEM supplemented with 10% FBS and 2 mM glutamine. The human breast carcinoma cell line, BT20 (Lastargues and Ozzello, 1958), was cultured in RPMI 1650 with 10% FBS. MCF-7 (Soule et al., 1973) breast carcinoma cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 10 μg/ml insulin, and 2 mM glutamine.

Hepatocytes were isolated from 250-400 g Sprague-Dawley rats (male or female), using the well-described two-step perfusion method (Berry and Friends, 1969; Gonias et al., 1989a). The rats were purchased from Hilltop Laboratories. The hepatic cells were released into Williams E medium containing 10% FBS. The preparations were enriched in hepatocytes by centrifugation so that non-parenchymal cells represented less than 2% of the final populations. Adherent hepatocyte cultures were greater than 98% viable as determined by trypan blue exclusion 2 hours after isolation.

Purified plasminogen and plasminogen-Sepharose

Plasminogen was purified from human plasma by affinity chromatography on lysine-Sepharose (Deutsch and Mertz, 1970). A linear gradient of EACA (0 to 15 mM) was used to elute the plasminogen. Final preparations contained mixtures of the two major glycoforms but were free of detectable plasmin, as determined by SDS-PAGE and S-2251 hydrolysis. Purified plasminogen (100 mg) was coupled to 10 g of CNBr-activated Sepharose CL-4B (Porath et al., 1973). Approximately 60% of the plasminogen bound to the resin.

Plasminogen binding to cells in culture

Isolated rat hepatocytes were plated in collagen-coated 12-well or 24-

well plates at a density of 500,000 cells/ml and cultured in Williams E medium with 10% FBS. After 2 hours, the cultures were washed three times with EBSS, 10 mM Hepes, 0.1% BSA (w/v), pH 7.4 (EHB-buffer). Plasminogen was radioiodinated to a specific activity of 1-2 μCi/μg with Iodobeads (Hall et al., 1990) or chloramine-T (Miles and Plow, 1985). The ^{125}I-plasminogen was then incubated with the hepatocyte cultures in EHB and aprotinin (70 μg/ml) for 4 hours at 4°C. EACA (10 mM) was added to some wells. EACA and excess nonradiolabeled plasminogen are equally effective in inhibiting specific ^{125}I-plasminogen binding to cells in culture (Hajjar et al., 1986; Gonias et al., 1989a). ^{125}I-Plasminogen-binding experiments were terminated by washing the cultures 3 times with EHB. The cells were then lysed overnight in 0.1 M NaOH/1% SDS (w/v). Radioactivity in the lysates was determined in a gamma counter. An identical procedure was used to characterize plasminogen binding to HepG2 or breast carcinoma cells.

Plasma membrane preparation

Plasma membrane-enriched fractions of isolated hepatocytes were prepared according to the method of Fleischer and Kervina (1986) as modified by Wolf et al. (1992). All fractionation steps were performed in medium supplemented with 1 mM PMSF, 70 μg/ml aprotinin, 30 μg/ml leupeptin, 0.2 μg/ml E-64, and 1 mM EDTA. The complete procedure yielded subcellular fractions enriched in nuclei (N), cytoplasm (C), mitochondria (M), and plasma membranes (P). Intermediate filament proteins are typically recovered in the P and N fractions (Hubbard and Ma, 1983).

SDS-PAGE and ligand blotting

Whole-cell homogenates, subcellular fractions, and purified proteins were subjected to SDS-PAGE (Laemmli, 1970). Resolved proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore) using a Hoefer Transphor apparatus (2 hours, 0.5 amp). The membranes were then stained with Coomassie Blue R250 (Bio-Rad, CA) or blocked with 5% nonfat dried milk in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), for ligand blotting or western blot analysis. To insure complete transfer, the original gel slab was stained with Coomassie Blue R250 as well.

Blocked PVDF membranes were rinsed twice with PBS and 0.1% (v/v) Tween-20 (PBS-T). ^{125}I-Plasminogen (10-20 nM) was then incubated with the membranes in PBS-T and 70 μg/ml aprotinin for 45 minutes. The blots were rinsed 3 times for 15 minutes in PBS-T, dried and autoradiographed. In control experiments, ^{125}I-plasminogen was incubated with the PVDF membranes in the presence of EACA.

Plasminogen-affinity chromatography

Enriched plasma membranes (P fraction) were solubilized in 10 mM sodium deoxycholate, pH 8.4, for 2 hours at 4°C and then subjected to centrifugation for 15 minutes at 10^5 ***g*** in a Beckman airfuge. The insoluble pellet and the supernatant were analyzed by SDS-PAGE and ^{125}I-plasminogen-ligand blotting. The detergent-insoluble pellet was extracted with 6 M urea for 4 hours at 25°C and then dialyzed against 10 mM sodium deoxycholate. The resulting solution and the original detergent-soluble portion of the P-fraction were separately subjected to affinity chromatography on plasminogen-Sepharose. Associated proteins were eluted from plasminogen-Sepharose with 15 mM EACA in 10 mM sodium deoxycholate. Fractions were analyzed by SDS-PAGE with silver staining.

Micro-sequence analysis of the major plasminogen-binding protein

The major plasminogen-binding protein was eluted from PVDF and subjected to amino-terminal sequence analysis using an Applied Biosystems 470A Gas-Phase Sequencer equipped with a model 120A on-line analyzer. Since initial analysis suggested a blocked amino terminus, the binding protein was treated with 150 mM cyanogen bromide (CNBr) in 70% formic acid while adherent to PVDF. Fragments were eluted with 1% (v/v) Triton X-100, 2% SDS, 50 mM Tris, pH 9.5, and then re-subjected to SDS-PAGE and electroblotting. New products were identified by Coomassie staining and subjected to amino-terminal sequence analysis. Results were compared with known sequences in GenBank using the Fasta Program (Pearson, 1994).

Immunofluorescence microscopy

Hepatocytes were adhered to collagen-coated 30 mm glass coverslips for 2 hours at 37°C and then studied immediately in immunofluorescence microscopy experiments. HepG2, BT20, and MCF-7 cells were cultured on coverslips for at least 48 hours. All cultures were washed with EHB buffer and incubated with various primary antibodies at 1/100-1/500 dilution in EHB for 2 hours at 4°C. The cells were then washed three times with EHB, incubated with FITC-labelled goat anti-mouse IgG (1/1000 dilution) for 2 hours at 4°C, rinsed three times again, and fixed in 3.7% paraformaldehyde or freshly prepared, ice-cold buffered-formaldehyde. Cellular immunofluorescence was imaged using an Olympus 3H2 Microscope with a dual wavelength cube. In control experiments, mouse nonimmune IgG was substituted for primary antibody or primary antibody was omitted.

Electron microscopy

Primary hepatocytes, breast carcinoma cells, and HepG2 cells were cultured on 10 mm plastic coverslips, as described for the immunofluorescence microscopy experiments. The cells were brought to 4°C and incubated with primary antibodies for 1 hour. At this point, the cells were incubated with either Protein A adsorbed to colloidal gold (100 Å) or anti-mouse IgG adsorbed to colloidal gold (100 Å) for 2 hours at 4°C. The cells were fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.5, for 15 minutes. This fixative rapidly reacts with the lipid in plasma membranes and limits plasma membrane vesiculation (Hasty and Hay, 1978). After washing with PBS, the cells were further fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.5, for 8 hours at 4°C. The cells were then serially dehydrated in acetone and embedded in EPON 812 for polymerization at 60°C overnight. Embedded coverslips were thin-sectioned on-side. Sections were transferred to 150 mesh nickel grids and viewed on a Zeiss 902 electron microscope.

RESULTS

Plasminogen binding to cells in culture

In initial studies, we confirmed that hepatocytes in primary culture, HepG2, BT20, and MCF-7 cells bind plasminogen specifically. As expected, EACA displaced greater than 80% of the binding through the entire ^{125}I-plasminogen concentration range with each cell type. Scatchard transformations were linear with correlation coefficients of 0.90-0.98 (not shown). K_d and B_{max} values were derived assuming a single-K_d model (Table 1) and are consistent with previously reported studies of plasminogen binding to cells in culture (as reviewed above).

Characterization of plasminogen-binding proteins in the hepatocyte P-fraction

Plasminogen binds specifically to a number of cellular

Table 1. Specific binding parameters for the interaction of ^{125}I-plasminogen with cells in culture

Cell type	K_d (μM)	B_{max} (per cell)
Rat hepatocytes	0.75(±0.1)	$9.7(\pm0.7)\times10^6$
HepG2	1.83(±0.6)	$4.3(\pm1.0)\times10^6$
BT20	1.05(±0.3)	$5.9(\pm1.0)\times10^6$
MCF-7	0.90(±0.3)	$1.9(\pm0.4)\times10^6$

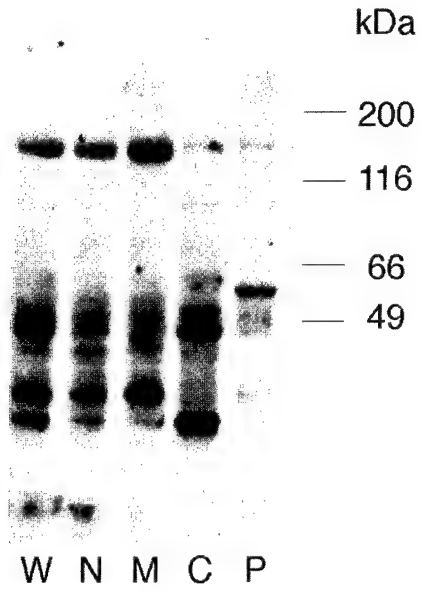


Fig. 1. ^{125}I-Plasminogen ligand blot of rat hepatocyte subcellular fractions. Lanes are labeled as follows: W, whole cell homogenate; N, nuclear fraction; M, mitochondrial fraction; C, cytoplasmic fraction; P, plasma membrane fraction. Each subcellular fraction has a unique set of plasminogen-binding proteins. The major plasminogen-binding species in the P fraction is the 59 kDa protein.

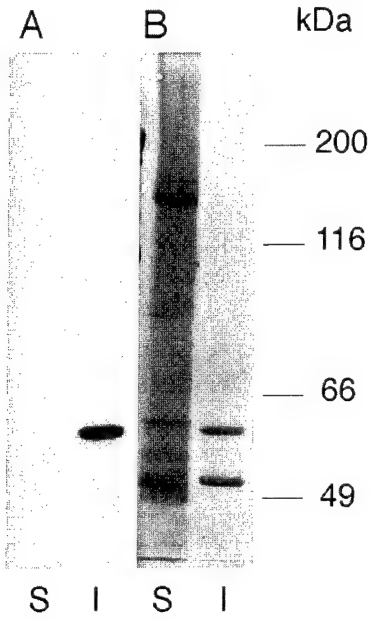


Fig. 2. ^{125}I-Plasminogen ligand blot and Coomassie-staining of detergent extracts of rat hepatocyte plasma membranes. (A) ^{125}I-plasminogen ligand blot of the detergent-soluble (S) and insoluble (I) fractions of the plasma membrane preparation. The detergent was 10 mM sodium deoxycholate. (B) Coomassie-stained blot of the same 'S' and 'I' fractions. The majority of the P-fraction is soluble in sodium deoxycholate. Two major bands remain in the detergent-insoluble fraction, including the 59 kDa plasminogen-binding protein.

proteins, including some that are intracellular and not available unless the cell is permeabilized. To identify plasminogen-binding proteins that may be significant in intact cells, hepatocytes were subjected to subcellular fractionation. Plasminogen-binding proteins in each fraction were compared by ^{125}I-plasminogen ligand blotting. Fig. 1 shows a representative ligand blot in which equal amounts of protein (100 μg) from the various subcellular fractions were compared. Each subcellular fraction demonstrated a distinct and consistent fingerprint of plasminogen-binding proteins. The primary plasminogen-binding protein in the P-fraction had a mass of 59 kDa. ^{125}I-Plasminogen binding to this band was completely inhibited by a 100-fold molar excess of nonradiolabeled plasminogen or 15 mM EACA (results not shown).

The 59 kDa plasminogen-binding protein was not detected in the M or C fractions, in five separate experiments. In whole cell homogenates and N-fractions, the 59 kDa protein either was detected only with prolonged autoradiograph exposure time (as was the case for the preparation shown in Fig. 1) or was present as a distinct but minor band compared with other plasminogen-binding species (as observed in other experiments not shown). These results suggest any of the following possibilities: (i) the 59 kDa protein is selectively recovered in the P-fraction; (ii) the plasminogen-binding activity of the 59 kDa protein is less stable in other fractions; and/or (iii) the other subcellular fractions contain a higher proportion of other plasminogen-binding proteins that are excluded from the P-fraction.

Characterization of the 59 kDa protein

The P-fraction was treated with various detergents, including 1% Triton X-100, 25 mM CHAPS, 35 mM β-D octylglucoside, and 10 mM sodium deoxycholate. Detergent-soluble and -insoluble fractions were incubated in SDS at 95°C for 5 minutes and subjected to SDS-PAGE and ^{125}I-plasminogen ligand blotting. Each of the detergents solubilized the majority of the P-fraction proteins; however, the 59 kDa protein remained in the detergent-insoluble fraction. Fig. 2 shows a representative solubilization experiment with 10 mM sodium deoxycholate. In this experiment, 100 μg of P-fraction were incubated with detergent for 2 hours and then subjected to centrifugation at 10^5 ***g***. The entire contents of the pellet (I) and supernatant (S) were subjected to SDS-PAGE so that the amount of each protein in the gel would approximate the proportion of that protein in the original P-fraction. As shown in the Coomassie-stained PVDF blot (B), the sodium deoxycholate-insoluble fraction contained two major proteins. One of these proteins was the 59 kDa species; this component was the only major ^{125}I-plasminogen-binding protein identified in either detergent solubilization fraction (A). The detergent-soluble fraction included a minor ^{125}I-plasminogen-binding species with an apparent mass of 150 kDa; this species was barely visible in the ligand blot without prolonged autoradiograph exposure time. An equivalently sized species was also apparent in the ligand blot of intact P-fraction shown in Fig. 1.

In ^{125}I-plasminogen ligand blotting experiments, P-fraction components were exposed to SDS. Therefore, we considered the possibility that the P-fraction contains plasminogen-binding proteins that are inactivated by SDS. In eight separate experiments, 5-15 mg of P-fraction were solubilized with the various detergents listed above. The detergent-soluble fractions were subjected to plasminogen-affinity chromato-

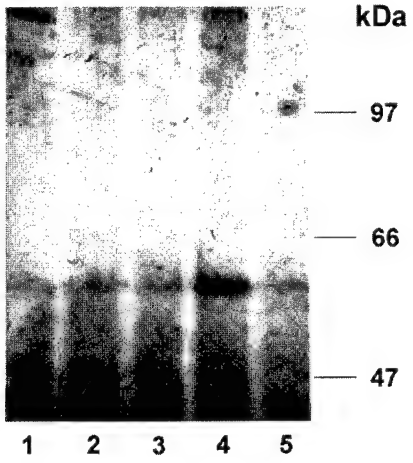


Fig. 3. Plasminogen-binding proteins isolated from the rat hepatocyte P-fraction by affinity chromatography. Lanes are labeled as sequential elution fractions, recovered after adding 15 mM EACA to the running buffer. Fraction 4 contains the major plasminogen-binding protein with a mass of 59 kDa.

graphy. EACA (15 mM) was used to elute bound proteins; elution fractions were analyzed by SDS-PAGE and silver-staining. A significant plasminogen-binding protein was not identified in these experiments.

Affinity chromatography was then performed to identify plasminogen-binding proteins in the detergent-insoluble portion of the P-fraction. Sodium deoxycholate (10 mM) was used to solubilize hepatocyte P-fraction. After centrifugation, the pellet was dissolved in 6 M urea and dialyzed back into 10 mM sodium deoxycholate without significant precipitation. The preparation was then subjected to plasminogen-affinity chromatography. Successive elution fractions, obtained after adding 15 mM EACA to the running buffer, are shown in Fig. 3. The 59 kDa protein bound to plasminogen-Sepharose and was the only major protein recovered in the EACA-eluent. These studies confirm and extend the results of the ligand blotting experiments by demonstrating that the 59 kDa protein binds plasminogen without exposure to SDS or immobilization on PVDF. Furthermore, the results of the chromatography experiments, performed with detergent-soluble and -insoluble fractions, indicate that the 59 kDa protein is the major plasminogen-binding protein in hepatocyte plasma membranes.

Identification of the 59 kDa protein

To identify the 59 kDa protein, sodium deoxycholate-insoluble material was isolated from the P-fraction. The 59 kDa protein was further purified by SDS-PAGE, transferred to PVDF and eluted for amino-terminal sequence analysis. Since the N terminus was apparently blocked, CNBr fragmentation was performed while the protein was adherent to the PVDF. The products were then eluted and subjected to SDS-PAGE and electroblotting. A major band at 12 kDa was isolated and subjected to amino-terminal sequence analysis, yielding a major and a secondary sequence. The major sequence, including the inferred N-terminal methionine residue, was MSTSGPRAFS. Amino acid yield ranged from 1.2 to 2.0 pmol, with the exception of the Ser residues that were recovered at lower yield, as expected. A search for homologous sequences was conducted in GenBank, using the Fasta program (Pearson, 1994). The major sequence was identical with that of mouse CK 8 (residues 12-21). Other significant homologies were detected exclusively with CK 8 from other species, including human (9/10) and potoroo (7/10).

The secondary sequence was present at approximately 25% of the molar yield of the major sequence and consisted of MDxIIAEVRA (a secondary amino acid was not clearly resolved at position three). The secondary sequence was 100% identical with amino acids 263-272 of mouse CK 8 and 89% identical with the comparable region of human CK 8. Significant homologies with proteins other than CK were not identified.

The identification of the plasminogen-binding protein as CK 8, or a CK 8-like protein, is consistent with the mass of the protein determined by SDS-PAGE and with the unique solubility properties of the protein, as determined by incubation with various detergents (Steinert and Roop, 1988). In hepatocytes and in other epithelial cells, CK 8 is typically recovered with CK 18 (Franke et al., 1981a,b), a slightly smaller protein that was observed in the detergent-insoluble fraction in Fig. 2. It is known that some CK 8 will partition into the plasma membrane fraction during subcellular fractionation (Hubbard and Ma, 1983). Furthermore, CK 8 is unique amongst the cytokeratins in that it has a C-terminal lysine that is conserved across species lines (Morita et al., 1988; Leube et al., 1986). As mentioned above, C-terminal lysine residues form a key element of most plasminogen-binding domains. To confirm the results of the microsequencing experiments, the detergent-insoluble portion of the hepatocyte P-fraction was subjected to western blot analysis with PCK-26, as previously described. The 59 kDa band bound antibody; no other immunopositive bands were detected (results not shown).

Immunofluorescence microscopy

The ability of CK 8 or a CK 8-like protein to bind plasminogen is significant, since CK 8 may be released by epithelial cells into the pericellular spaces and into the blood where plasminogen is present (Chan et al., 1986). CK 8 release from cells may be significantly increased in cancer (Bjorklund and Bjorklund, 1983). It has also been suggested that CK 8 is present on the external surfaces of breast carcinoma cells (Godfroid et al., 1991; Donald et al., 1990). If these reports are correct, then cell-surface CK 8 may function as a cellular plasminogen receptor; however, the presence of CK 8 on cell surfaces has been disputed (Riopel et al., 1993). Therefore, we undertook studies to determine whether hepatocytes and other epithelial cells express cell surface CK 8. The techniques used included immunofluorescence microscopy and immunoelectron microscopy. The advantage of these techniques is the ability to determine whether an antigen is distributed homogeneously across an entire population of cells or exclusively by a small subpopulation of cells.

To stain only cell-surface CK 8, CK 8-specific antibodies and secondary antibody were incubated with viable cells at 4°C in physiological buffers that contained BSA. Since all of the incubations were conducted with adherent cells on coverslips, the chance of cellular injury due to culture manipulation was minimized. Cells were not fixed until after all antibody incubations were completed.

Immunofluorescence microscopy studies of hepatocytes

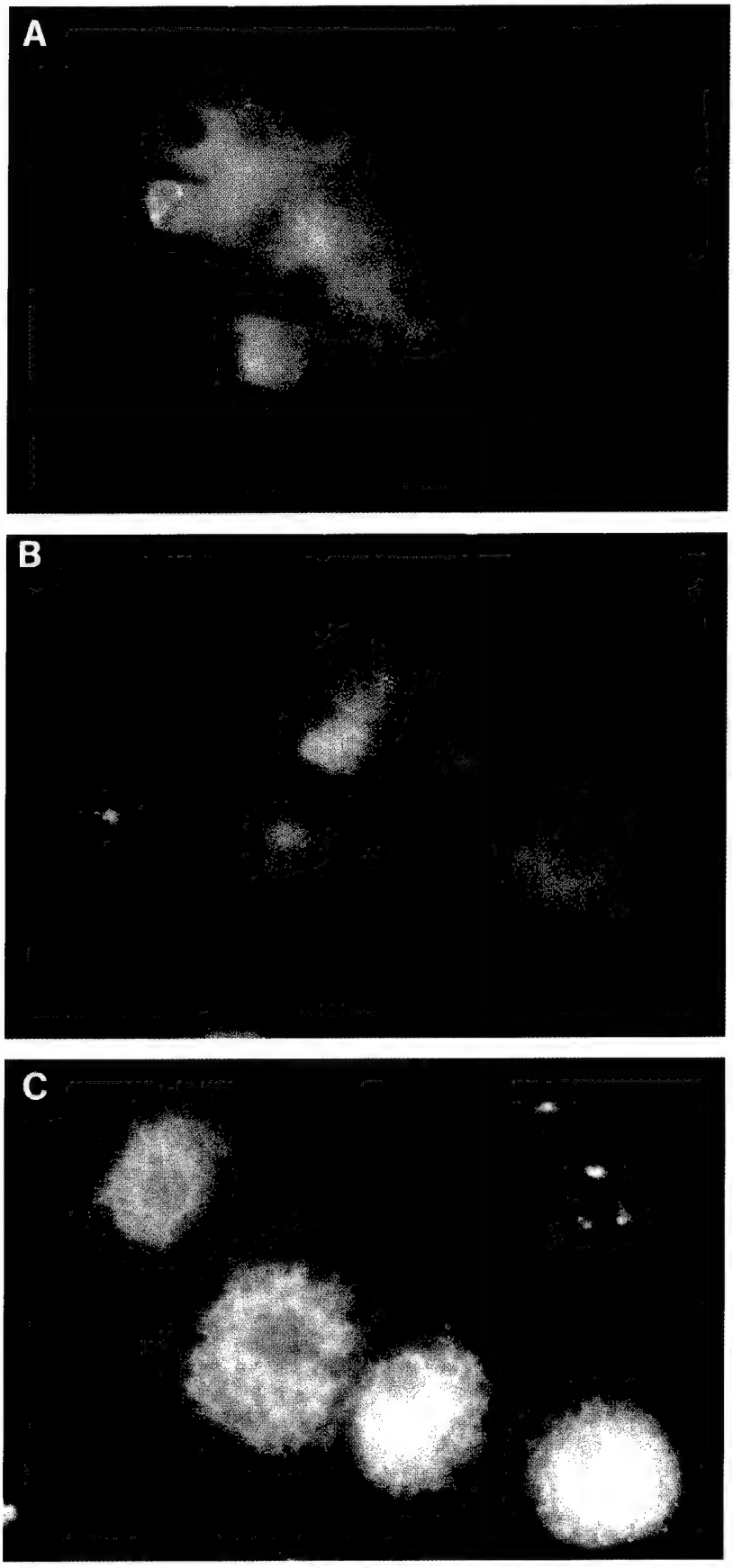


Fig. 4. Detection of CK 8 in viable, unpermeabilized hepatocytes by immunofluorescence microscopy. Primary rat hepatocytes were incubated with AB 6.01, followed by secondary antibody (FITC-anti-mouse IgG). To differentiate antibody binding from hepatocyte autofluorescence, the cells were imaged using a dual wavelength cube, exciting at both FITC and Texas Red wavelengths. Under these conditions, cytoplasmic autofluorescence appears red-white.
(A) AB 6.01 labeling of a hepatocyte-cluster.
(B) Individual hepatocytes are more prevalent.
(C) Incubation with primary antibody was omitted; only cytoplasmic autofluorescence is seen.

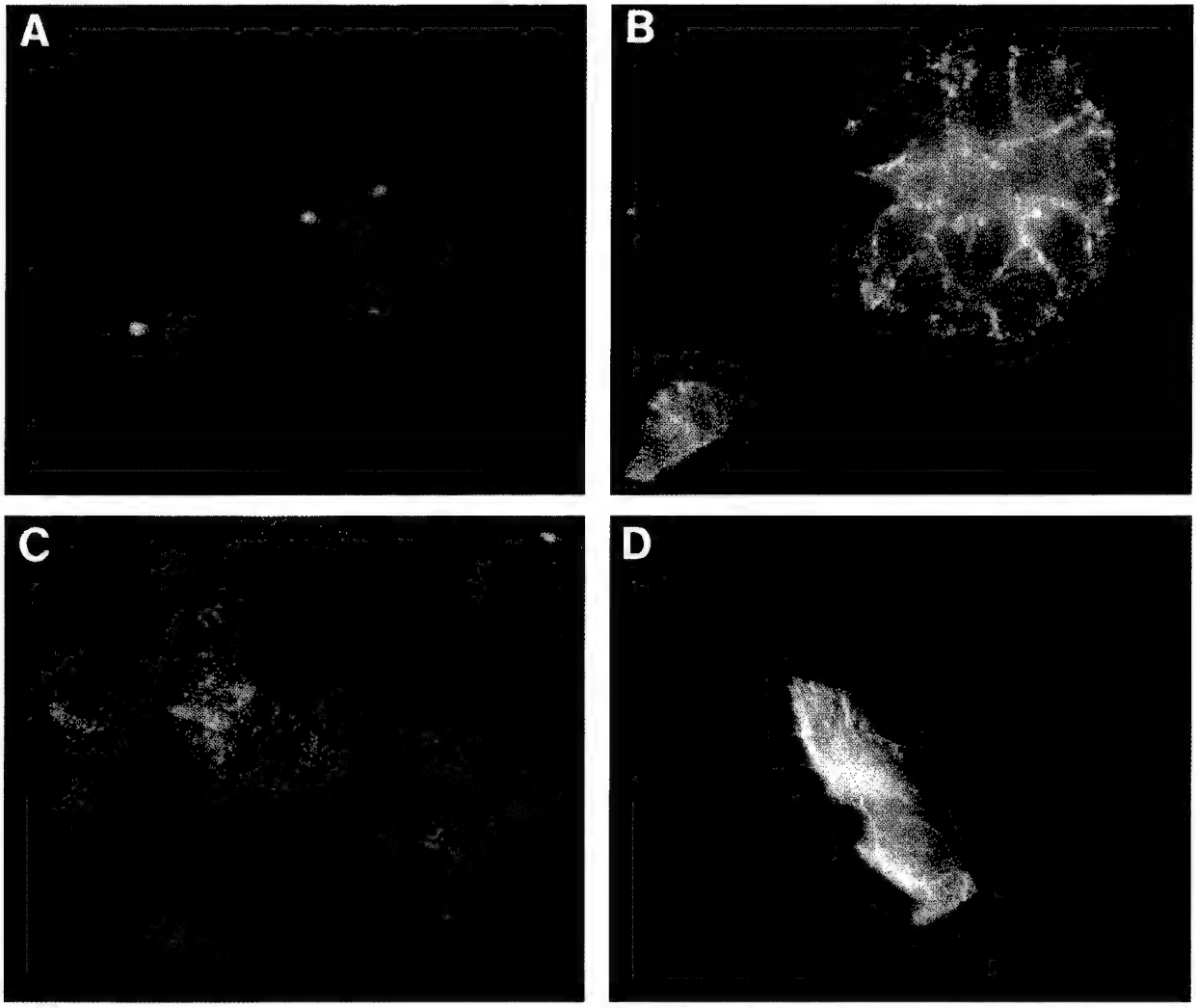


Fig. 5. Detection of CK 8 in viable, unpermeabilized HepG2 human hepatocellular carcinoma cells by immunofluorescence microscopy. (A-C) The cells were incubated with AB 6.01 and secondary antibody prior to fixation. (D) A typical HepG2 cell labeled with AB 6.01 after fixation and permeabilization with 0.02% saponin. The basket-like, perinuclear pattern of intracytoplasmic CK staining in D is readily distinguished from the pattern of cell-surface staining in A-C.

were first performed using AB 6.01 (Fig. 4). A punctate staining pattern was distributed uniformly over the entire surface of every cell. The extent of staining and the pattern were not affected by whether the cells were clustered in aggregates, as in panel A, or isolated, as in panel B. When the primary antibody was omitted, or when nonimmune mouse IgG was substituted for AB 6.01, only the inherent autofluorescence of the hepatocytes was detected (panel C). Similar punctate staining was observed using antibody PCK-26 (results not shown), which is specific for CK 8 in hepatocytes, since these cells do not express CK 5 or CK 6 (Moll et al., 1982; Franke et al., 1981a,b).

Since hepatocytes might be slightly damaged during isolation for primary culture, the immunofluorescence microscopy studies were extended by studying cell lines. Cultures of HepG2 cells were studied 48 hours after transfer to coverslips using AB 6.01. The labeling was similar to that observed with hepatocytes, except for the slightly finer punctate pattern in the HepG2 cells (Fig. 5). In panels A and B, the plane of focus is low near the coverslip. Therefore, the immunofluorescence is seen in association with the edges of the cells, and at cell-cell contacts. In panel C, the plane of focus is near the apical surfaces of some cells, so that the punctate pattern of plasma membrane staining is more apparent. No immunofluorescence was observed when primary antibody was omitted. For comparison, some HepG2 cells were permeabilized with 0.02% saponin prior to incubation with primary antibody. The typical basket-like pattern of intracellular cytokeratin staining (panel D) was significantly different from that observed with viable, unpermeabilized cells.

Many of the previous conflicting studies regarding cell-surface CK 8 were performed with cultures of breast carcinoma cells (Godfroid et al., 1991; Donald et al., 1990). Therefore, we examined surface exposure of CK 8 in BT20 and MCF-7 breast carcinoma cells. Fig. 6 presents a composite of experiments performed with BT20 cells. AB 6.01 was used in panels A and C; M20 was used in panel B. The pattern of staining was comparable to that observed with the HepG2 cells and

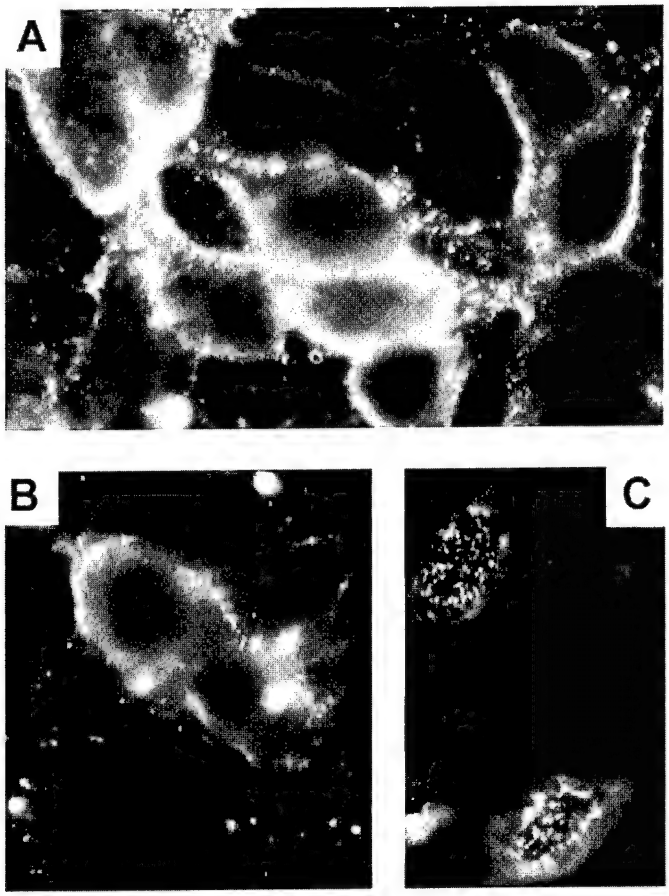


Fig. 6. Detection of CK 8 in viable, unpermeabilized BT20 human breast carcinoma cells by immunofluorescence microscopy. BT20 cells were incubated with AB 6.01 in A and C, or with M20 in B, followed by FITC-conjugated secondary antibody. All cells show diffuse punctate cell surface staining. In A and B, the plane of focus is near the coverslip to demonstrate peripheral, plasma-membrane-associated immunofluorescence. In C, the plane of focus is near the apical surface of the cell and shows the fine punctate pattern of immunofluorescence.

hepatocytes. The plane of focus in panels A and B is low, near the coverslip, demonstrating the plasma membrane distribution of immunofluorescence. Panel C shows punctate staining of apical cell surfaces. Equivalent results were obtained with MCF-7 cells (results not shown). Staining was not observed when primary antibody was omitted or when primary antibody was replaced with nonimmune mouse IgG. Furthermore, the pattern and intensity of staining remained unchanged when the cells were fixed prior to addition of secondary antibody (results not shown).

As a further control for the immunofluorescence microscopy experiments, MCF-7 and BT20 cells were examined using monoclonal antibody directed against the intracellular antigen, p120-GAP. Intense perinuclear fluorescence was observed in cells permeabilized with 0.02% saponin. By contrast, unpermeabilized cells demonstrated no immunofluorescence (results not shown).

Immunoelectron microscopy

To confirm that CK 8 or a CK 8-like antigen is associated with the external surfaces of cells, immunoelectron microscopy experiments were performed. Primary antibody and colloidal gold adducts were incubated with viable cells at 4°C. Under these conditions, cell-associated gold should, hypothetically, indicate the presence of cell surface-bound primary antibody. Fig. 7 presents a composite of imaging studies with hepatocytes, HepG2 cells, BT20 and MCF-7 breast carcinoma cells. In A and B, hepatocytes were incubated with PCK-26. Colloidal gold was observed on the cell surfaces, primarily in association with microvilli and adjacent plasma membranes. No colloidal gold was observed in association with the intracellular spaces of the hepatocytes, suggesting that the cellular membranes were intact and that neither the primary antibody nor colloidal gold had access to the large intracellular pools of CK 8. When hepatocytes were incubated with colloidal gold adduct without prior incubation with PCK-26, essentially no colloidal gold was observed in association with the cell surfaces (results not shown).

The HepG2 cells and breast carcinoma cells were studied using AB 6.01. With each of these epithelial cells, the pattern of immunogold labeling was similar. Colloidal gold was localized exclusively to the cell surface and frequently observed in association with microvilli. As shown in C, large amounts of colloidal gold were observed in association with cellular projections near HepG2 cell-cell junctions. Omission of primary antibody consistently eliminated binding of colloidal gold-adduct to the cell surfaces. Clustering of colloidal gold particles, such as that observed in F, should be interpreted with caution, since the cells were exposed to Protein A-colloidal gold prior to fixation. The colloidal gold-adduct may promote clustering of antigen if the antigen is mobile in the plasma membrane.

In order to demonstrate that large amounts of antigen could be detected by immunoelectron microcopy if the cells were permeabilized, MCF-7 cells were incubated with AB 6.01 and Protein A-colloidal gold, post-embedment (H). The colloidal gold is observed in association with the cells, primarily overlying cytoplasm, as expected.

DISCUSSION

The role of plasmin in tumor invasion and other processes that require cellular migration has been studied extensively (Ellis and Dano, 1992; Vassalli et al., 1991). In this and related systems, association of plasminogen with the cell surface is probably critical. While recent studies have made significant advances in identifying important plasminogen receptors on various cells, epithelial cells have received little attention. These cells are important, since a large variety of common malignancies are of the epithelial cell-derived carcinoma family.

In this investigation, the primary plasminogen-binding protein in the plasma membrane fraction of hepatocytes was identified. This protein was CK 8 or a very closely related homologue of CK 8, as suggested by the molecular mass of the protein, the insolubility of the protein in various detergents, the primary sequence in two different regions of the structure, and the reactivity of the protein with an antibody that should be specific for CK 8 in western blots of hepatocyte extracts. The affinity of CK 8 for plasminogen is predicted by the presence

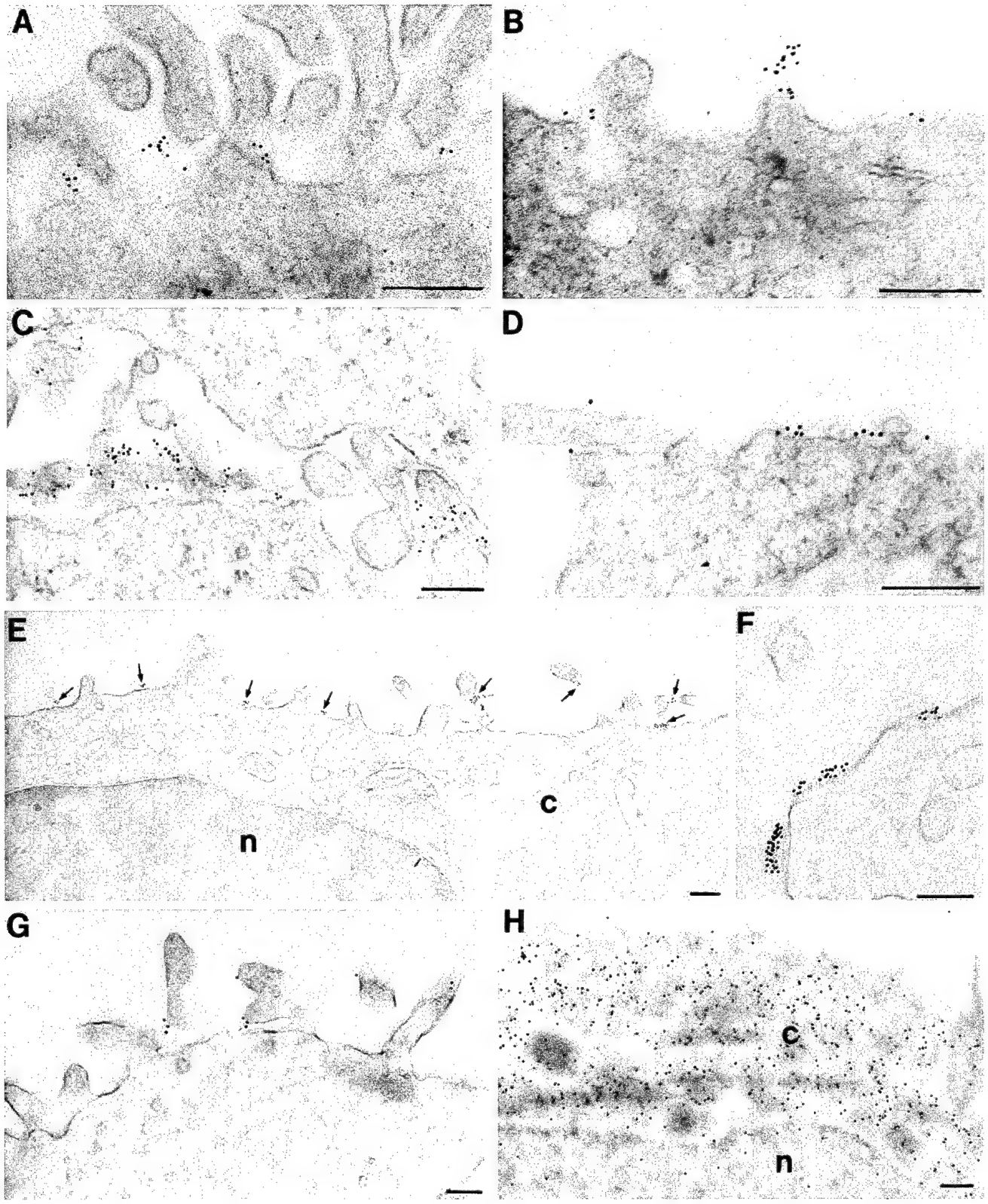


Fig. 7. Immunoelectron microscopy analysis of CK 8 in viable, unpermeabilized rat hepatocytes, HepG2, BT20 and MCF-7 cells. Rat hepatocytes, shown in A and B, were incubated with PCK-26, followed by anti-mouse IgG-colloidal gold adduct. HepG2 cells are shown in C and D, BT20 cells in E and F, and MCF-7 cells are shown in G. The cells in these panels were incubated with AB 6.01, followed by Protein A-colloidal gold adduct. The arrows in E indicate clusters of colloidal gold. In H, MCF-7 cells were incubated with AB 6.01 and Protein A-colloidal gold adduct post-embedment. This procedure exposes intracytoplasmic CK to antibody. c, cytoplasm; n, nucleus. Bars, 200 nm.

of a conserved C-terminal lysine in the CK 8 sequence. Although this is the first description of this novel activity for CK 8, the significance of our observation is dependent on the extent to which plasminogen has access to CK 8.

CK 8 is one of at least 21 related cytokeratins that form intermediate filaments in various epithelial cells and carcinoma cells; however, many cell types express only one acidic (type I) CK and one neutral-basic (type II) CK (Moll et al., 1982). An example is the hepatocyte, which expresses CK 8 and CK 18 (Franke et al., 1981a,b). CK 8-containing intermediate filaments associate extensively with the internal leaflet of the plasma membrane. CK 8 or CK 8 fragments have also been detected in conditioned culture medium (Chan et al., 1986) and in the serum and body fluids of patients with a variety of cancers (Bjorklund and Bjorklund, 1983). It is not clear whether the released CK in vivo is derived primarily from viable or dying cells. Nevertheless, this pool of CK 8 is present in physiological spaces that contain plasminogen. Therefore, it is feasible that CK 8 functions as a plasminogen-binding protein in vivo.

Previous studies have provided evidence for the presence of CK 8 on the external surfaces of cultured breast cancer cell lines (Godfroid et al., 1991; Donald et al., 1991). This association may be accomplished in several ways. It is feasible that CK 8 projects through the plasma membrane as part of a protein complex so the CK 8 does not require a transmembrane domain. CK 8 may associate with the plasma membrane after secretion by cells (Riopel et al., 1993). It has also been demonstrated that CK 8 covalently binds to plasma membrane lipids (Asch et al., 1993); these covalent conjugates may conceivably translocate to the outer membrane leaflet during normal membrane shedding. Our further investigation of this issue was prompted by the work of Riopel et al. (1993), which suggested that detection of cell-surface CK is artifactual. These investigators presented a convincing argument regarding the inadequacy of methods such as cell surface radioiodination for the demonstration of cell surface CK 8. Using such methods, even a rare injured or permeabilized cell might confound the data, since the amount of intracellular CK 8 greatly exceeds that which might be present on the cell surface.

The immunofluorescence microscopy studies presented here are the first of their kind with hepatocytes, HepG2 hepatocellular carcinoma cells and BT-20 breast carcinoma cells. MCF-7 cell immunofluorescence microscopy studies using antibody 6.01 were reported previously by Donald et al. (1991). We chose immunofluorescence microscopy for antibody detection instead of other techniques, such as FACS analysis, so that adherent cultures might be studied without using potentially damaging techniques to release cells. Our results suggest a similar pattern of punctate cell surface CK 8-antigen detection with each of the cell types examined. The protocols used in the immunofluorescence microscopy studies were designed so as to minimize the possibility of cellular injury or death. The uniform pattern of immunofluorescence through the entire population of cells in each preparation demonstrates that the antibody-accessible CK 8 is not contributed by a small subpopulation of damaged cells. Furthermore, the punctate pattern of fluorescence in the intact cells is readily distinguishable from that observed with permeabilized cells. Overall, three separate antibodies were used in the immunofluorescence studies with equivalent results: PCK-26 with hepatocytes; AB 6.01 with hepatocytes, HepG2 cells and breast carcinoma cells; and M20 with breast carcinoma cells. The use of multiple antibodies with well-characterized specificities minimizes the chance that we are detecting a cell-surface protein that is completely unrelated to CK 8. Furthermore, our western blot analyses of whole cell extracts from each of the studied cell types confirmed the expected specificities for each antibody (PCK-26, M20, AB 6.01) (results not shown). Therefore, the immunofluorescence studies strongly suggest the presence of CK 8 or a CK 8-like protein on the surfaces of viable epithelial cells.

Immunoelectron microscopy experiments were performed to confirm that our methods detect cell-surface antigen and not intracytoplasmic antigen. As with the immunofluorescence studies, electron microscopy revealed a similar pattern of antigen expression in all of the epithelial cells studied. Although colloidal gold was distributed across the entire cell surface, we always observed gold preferentially associated with microvilli and the plasma membranes adjacent to microvilli. The pattern of antigen detection by immunogold reported here in hepatocytes, HepG2, and breast carcinoma cells is similar to that reported by Donald et al. (1991) in squamous cell carcinoma.

Our finding that a cytoskeletal protein is located on the cell surface where it may function as an important receptor is not unique. α-Actin has been reported to be present on the surfaces of multiple cell types, including fibroblasts, smooth muscle cells and endothelium (Chen et al., 1978; Jones et al., 1979; Moroianu et al., 1993). Endothelial cell-surface α-actin may function as a receptor for angiogenin and thereby regulate angiogenesis (Moroianu et al., 1993).

The studies presented in this investigation do not permit a determination of the extent to which extracellular CK 8 contributes to the total specific plasminogen-binding capacity of the various cell types. The CK 8-specific antibodies used in this study were of the IgG_1 subtype and therefore all have carboxyl-terminal lysine residues. Although we have performed experiments demonstrating that M20 and PCK-26 substantially inhibit plasminogen binding to each of the cells under investigation (AB 6.01 was a weaker inhibitor, results not shown), Ellis and Dano (1993) demonstrated that IgG_1 antibodies bind plasminogen. Therefore, our results may be explained by antibody binding to plasminogen receptors on the cell surface or by plasminogen-antibody complex formation in solution. To resolve this problem, we are currently attempting to raise monoclonal antibodies specific for the C terminus of CK 8. If an appropriate hybridoma can be obtained, competition binding studies will be performed with Fab fragments.

Numerous studies have shown high levels of CK 8 in malignant cells. In transitional cell carcinoma and squamous cell carcinoma, CK 8 and CK 18 have been detected at increased levels by immunohistochemistry at the tumor invasion front (Schaafsma et al., 1991,1993). The most tumorigenic clones of SW 613-S cells express the highest levels of CK 8 mRNA (Modjtahedi et al., 1992). CK 8 is aberrantly induced in H-*ras* transformed epidermal cells (Diaz-Guerra et al., 1992). Furthermore, mouse L fibroblasts, which lack CK 8 and 18, show increased motility and penetration of Matrigel in vitro after transfection with CK 8 and CK 18 DNAs (Chu et al., 1993). Motility and Matrigel penetration are processes which are dependent on the cell-surface tumor invasion pro-

teinase cascade. Whether cells that express increased levels of CK 8 show an incremental increase in cell surface CK 8 which, by binding plasminogen, supports or accelerates cellular migration and invasion is currently under investigation.

This work was supported by grants HL45786 and DK46735 from the National Institutes of Health (USA) and by grant no. 498 from the Department of the Army (USA) Breast Cancer Research Program.

REFERENCES

Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* **282**, 615-6.

Asch, H. L., Mayhew, E., Lazo, R. O. and Asch, B. B. (1993). Lipids covalently bound to keratins of mouse mammary epithelial cells. *Biochem. Mol. Biol. Int.* **29**, 1161-1169.

Berry, M. N. and Friends, D. S. (1969). High yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* **43**, 506-520

Bjorklund, B. and Bjorklund, V. (1983). Specificity and basis of the tissue polypeptide antigen. *Cancer Detect. Prevent.* **6**, 41-50.

Burtin, P. and Fondaneche, M. C. (1988). Receptor for plasmin on human carcinoma cells. *J. Nat. Cancer Inst.* **80**, 762-765.

Campbell, P. G., Novak, J. F., Yanosick, T. B. and McMaster, J. H. (1992). Involvement of the plasmin system in the dissociation of the insulin-like growth factor-binding protein complex. *Endocrinology* **130**, 1301-1312.

Chan, R., Rossitto, P. V., Edwards, B. F. and Cardiff, R. D. (1985). Intracellular and extracellular keratins of human mammary epithelial cells. In *Monoclonal Antibodies and Breast Cancer* (ed. R. L. Ceriani), pp. 165-178. Nijhoff, Boston.

Chan, R., Rossitto, P. V., Edwards, B. F. and Cardiff, R. D. (1986). Presence of proteolytically processed keratins in the culture media of MCF-7 cells. *Cancer Res.* **46**, 6353-6359

Chen, L. B., Murray, A., Segal, R. A., Bushnell, A. and Walsh, M. L. (1978). Studies on intercellular LETS glycoprotein matrices. *Cell* **14**, 377-391.

Christensen, U. (1985). C-terminal lysine residues of fibrinogen fragments essential for binding to plasminogen. *FEBS Lett.* **182**, 43-46.

Chu, Y-W, Runyan, R. B., Oshima, R. G. and Hendrix, M. J. C. (1993). Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. *Proc. Nat. Acad. Sci. USA* **90**, 4261-4625.

Correc, P., Fondaneche, M. C., Bracke, M. and Burtin, P. (1990). The presence of plasmin receptors on three mammary carcinoma MCF-7 sublines. *Int. J. Cancer* **46**, 745-750.

Deutsche, D. G. and Mertz, E. T. (1970). Plasminogen: purification from human plasma by affinity chromatography. *Science* **170**, 1095-1096.

Diaz-Guerra, M., Haddow, S., Bauluz, C., Jorcano, J. L., Cano, A., Balmain, A. and Quintanilla, A. (1992). Expression of simple epithelial cytokeratins in mouse epidermal keratinocytes harboring Harvey ras gene alterations. *Cancer Res.* **52**, 680-687.

Donald, P. J., Cardiff, R. D., He, D. and Kendall, K. (1991). Monoclonal antibody-porphyrin conjugate for head and neck cancer: The possible magic bullet. *Otolaryngol. Head Neck Surg.* **105**, 781-787.

Ellis, V. and Dano, K. (1992). The urokinase receptor and the regulation of cell surface plasminogen activation. *Fibrinolysis* **6** (suppl.), 27-34.

Ellis, V. and Dano, K. (1993). Potentiation of plasminogen activation by an anti-urokinase monoclonal antibody due to ternary complex formation. *J. Biol. Chem.* **268**, 4806-4813.

Fleischer, S. and Kervina, M. (1986). Subcellular fractionation of rat liver. *Meth. Enzymol.* **31**, 6-41.

Fleury, V. and Anglés-Cano, E. (1991). Characterization of the binding of plasminogen to fibrin surfaces: the role of carboxyl-terminal lysines. *Biochemistry* **30**, 7630-7638.

Franke, W. W., Denk, H., Kalt, R. and Schmid, E. (1981a). Biochemical and immunological identification of cytokeratin proteins present in hepatocytes of mammalian liver tissue. *Exp. Cell Res.* **131**, 299-318.

Franke, W. W., Mayer, D., Schmid, E., Denk, H. and Borenfreund, E. (1981b). Differences of expression of cytoskeletal proteins in cultured rat hepatocytes and hepatoma cells. *Exp. Cell Res.* **134**, 345-365.

Garrod, D. R. (1993). Desmosomes and hemidesmosomes. *Curr. Opin. Cell Biol.* **5**, 30-40.

Godfroid, E., Geuskens, M., Dupressoir, T., Parent, I. and Szpirer, C. (1991). Cytokeratins are exposed on the outer surface of established human mammary carcinoma cells. *J. Cell Sci.* **99**, 595-607.

Gonias, S. L., Braud, L. L., Geary, W. A. and VandenBerg, S. R. (1989a). Plasminogen binding to rat hepatocytes in primary culture and to thin slices of rat liver. *Blood* **74**, 729-736.

Gonias, S.L., Young, W.W. and Fox, J.W. (1989b). Cleavage of recombinant murine interferon-γ by plasmin and miniplasmin. *J. Interferon Res.* **9**, 517-529.

Gonias, S. L. (1992). α_2-Macroglobulin: A protein at the interface of fibrinolysis and cellular growth regulation. *Exp. Hematol.* **20**, 302-311.

Hajjar, K. A., Harpel, P., Jaffe, E. and Nachman, R. L. (1986). Binding of plasminogen to cultured human endothelial cells. *J. Biol. Chem.* **261**, 11656-11662.

Hajjar, K. A., Jacovina, A. T. and Chacko, J. (1994). An endothelial cell receptor for plasminogen/tissue plasminogen activator. *J. Biol. Chem.* **269**, 21191-21197.

Hall, S. W., VandenBerg, S. R. and Gonias, S. L. (1990). Plasminogen carbohydrate side chains in receptor binding and enzyme activation: a study of C6 glioma cells and primary cultures of rat hepatocytes. *J. Cell. Biochem.* **43**, 213-227.

Hasty, D. L. and Hay, E. D. (1978). Freeze fracture studies of the developing cell surface. *J. Cell Biol.* **78**, 756-768.

He, C., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z. and Goldberg, G. I. (1989). Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. Nat. Acad. Sci. USA* **86**, 2636-2636.

Hubbard, A. L. and Ma, A. (1983). Isolation of rat hepatocyte plasma membranes. II. Identification of membrane associated cytoskeletal proteins. *J. Cell Biol.* **96**, 230-239.

Jones, P. A., Scott-Burden, T. and Gevers, W. (1979). Glycoprotein, elastin and collagen secretion by rat smooth muscle cells. *Proc. Nat. Acad. Sci. USA* **76**, 353-357.

Kanalas, J. J. and Makker, S. P. (1991). Identification of the rat Heymann nephritis autoantigen (GP330) as a receptor site for plasminogen. *J. Biol. Chem.* **266**, 10825-10829.

Laemmli, U. K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LaMarre, J., Vasudevan, J. and Gonias, S. L. (1994). Plasmin cleaves betaglycan and releases a 60 kDa transforming growth factor β-complex from the cell surface. *Biochem. J.* **302**, 199-205.

Lastargues, E. Y. and Ozzello, L. (1958). Cultivation of human breast carcinomas. *J. Nat. Cancer Inst.* **21**, 1131-1147.

Leube, R. E., Bosche, F. X., Romano, V., Zimbelmann, R., Holfer, H. and Franke, W. W. (1986). Cytokeratin expression in simple epithelial. *Differentiation* **33**, 69-85.

Liotta, L. A., Goldfarb, R. H., Brundage, R., Siegal, G. P., Teranova, V. and Gabrisa, S. (1981). Effect of plasminogen activator, plasmin and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res.* **41**, 4629.

Lyons, R. M., Keski-Oja, J. and Moses, H. L. (1988). Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J. Cell Biol.* **106**, 1659-1665.

Miles, L. A. and Plow, E. F. (1985). Binding and activation of plasminogen on the platelet surface. *J. Biol. Chem.* **260**, 4303-4311.

Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. (1991). Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* **30**, 1682-1691.

Modjtahedi, N., Frebourg, T., Fossar, N., Lavialle, C., Cremisi, C. and Brison, O. (1992). Increased expression of cytokeratin and ferritin-H genes in tumorigenic clones of the SW 613-S human colon carcinoma cell line. *Exp. Cell Res.* **201**, 74-82.

Moll, R., Franke, W. W. and Schiller, D. L. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**, 11-24.

Morita, T., Tondella, M. L., Takemoto, Y., Hashido, K., Ichinose, Y., Nozaki, M. and Matsushiro, A. (1988). Nucleotide sequence of mouse EndoA cytokeratin cDNA reveals polypeptide characteristics of the type-II keratin subfamily. *Gene* **68**, 109-117.

Moroianu, J., Fett, J. W., Riordan, J. F. and Vallee, B., L. (1993). Actin is a surface component of calf pulmonary artery endothelial cells in culture. *Proc. Nat. Acad. Sci. USA* **90**, 3815-3819.

Owaribe, K., Nishizawa, Y. and Franke, W. W. (1991). Isolation and

characterization of hemidesmosomes from bovine corneal epithelial cells. *Exp. Cell Res.* **192**, 622-630.

Parkkinen, J. and Rauvala, H. (1991). Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. *J. Biol. Chem.* **266**, 16730-16735.

Pasche, B., Ouimet, H., Francis, S. and Loscalzo, J. (1994). Structural changes in platelet glycoprotein IIb/IIIa by plasmin: determinants and functional consequences. *Blood* **83**, 404-414.

Pearson, W. R. (1994). Using the Fasta program to search protein and DNA sequence databases. *Meth. Mol. Biol.* **25**, 365-389.

Plow, E. F., Freaney, D. E., Plescia, J. and Miles, L. A. (1986). The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol.* **103**, 2411-2420.

Ponting, C. P., Marshall, J. M. and Cederholm-Williams, S. A. (1992). Plasminogen: a structural review. *Blood Coagulation Fibrinolysis* **3**, 605-614.

Porath, J., Aspberg, K., Drevin, H. and Axen, R. (1973). Preparation of cyanogen bromide-activated agarose gels. *J. Chromatogr.* **86**, 53-56.

Riopel, C. L., Butt, I. and Omary, M. B. (1993). Method of cell handling affects leakiness of cell surface labeling and detection of intracellular keratins. *Cell Motil. Cytoskel.* **26**, 77-87.

Schaafsma, H. E., Ramaekers, F. C. S., van Muijen, G. N. P., Robben, H., Lane, E. B., Leigh, I. M., Ooms, E. C. M., Schalken, J. A., van Moorselaar, R. J. A. and Ruiter, D. J. (1991). Cytokeratin expression patterns in metastatic transitional cell carcinoma of the urinary tract. *Am. J. Pathol.* **139**, 1389-1400.

Schaafsma, H. E., van der Velden, L. A., Manni, J. J., Peters, H., Link, M., Ruiter, D. J. and Ramaekers, F. C. S. (1993). Increased expression of cytokeratins 8, 18 and vimentin in the invasion front of mucosal squamous cell carcinoma. *J. Pathol.* **170**, 77-86.

Soule, H. D., Vasquez, J., Long, A., Albert, S. and Brennan, M. D. (1973). A human cell line from pleural effusion derived from a breast carcinoma. *J. Nat. Cancer Inst.* **51**, 1409-1416.

Steinert, P. M. and Roop, D. R. (1988). Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* **57**, 593-625.

Turner, J. S., Redpath, G. T., Humphries, J. E., Gonias, S. L. and VandenBerg, S. R. (1994). Plasmin modulates the thrombin-evoked calcium response in C6 glioma cells. *Biochem. J.* **297**, 175-179.

Vassalli, J.-D., Sappino, A.-P. and Belin, D. (1991). The plasminogen activator/plasmin system. *J. Clin. Invest.* **88**, 1067-1072.

Weber, K., Osborn, M., Moll, R., Wiklund, B. and Luning, B. (1984). Tissue polypeptide antigen (TPA) is related to the non-epidermal keratins 8, 18 and 19 typical of simple and non-squamous epithelia: re-evaluation of a tumor marker. *EMBO J.* **3**, 2707-2714.

Wolf, B. B., Lopes, M. B., VandenBerg, S. R. and Gonias, S. L. (1992). Characterization and immunohistochemical localization of α_2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. *Am. J. Path.* **141**, 37-42.

(Received 30 September 1994 - Accepted 11 November 1994)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 41, Issue of October 11, pp. 25684–25691, 1996

Printed in U.S.A.

Cell-surface Cytokeratin 8 Is the Major Plasminogen Receptor on Breast Cancer Cells and Is Required for the Accelerated Activation of Cell-associated Plasminogen by Tissue-type Plasminogen Activator*

(Received for publication, January 31, 1996, and in revised form, July 18, 1996)

Todd A. Hembrough‡, Li Li§, and Steven L. Gonias‡§¶

From the Departments of ‡Biochemistry and §Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Cytokeratin 8 (CK 8) has been identified on the external surfaces of viable, unpermeabilized epithelial cells (Hembrough, T. A., Vasudevan, J., Allietta, M. M., Glass, W. F., and Gonias, S. L. (1995) *J. Cell Sci.* 108, 1071–1082). In this study, we demonstrated that CK 8 is the major plasminogen-binding protein in plasma membrane fractions isolated from three breast cancer cell lines, BT20, MCF-7, and MDA-MB-157. To assess the function of CK 8 as a plasminogen receptor, monoclonal antibody 1E8 was raised against the carboxyl-terminal 12 amino acids of CK 8. The 1E8 epitope was present on the external surfaces of breast cancer cells, as determined by immunofluorescence microscopy. ^{125}I-1E8 bound to MCF-7 cells; the maximum binding capacity (1.5×10^6 sites per cell) was comparable with that determined for plasminogen. When MCF-7 cells were incubated with Fab fragments of 1E8, specific ^{125}I-plasminogen binding was decreased up to 82%. Specific plasminogen binding was decreased up to 67%, even when the unbound 1E8 Fab was removed by washing the cells prior to adding ^{125}I-plasminogen. Preincubation with 1E8 Fab decreased plasminogen binding to BT20 and MDA-MB-157 cells, although to a lesser extent than with MCF-7 cells. Plasminogen activation by tissue-type plasminogen activator was greatly accelerated, due to a large decrease in K_m, when the plasminogen was bound to MCF-7 cells. Pretreatment with 1E8 Fab decreased the rate of plasminogen activation by up to 83%, implicating CK 8 in the MCF-7 cell-accelerated reaction. These studies identify cell-surface CK 8 as a major plasminogen receptor in breast cancer cells and as a required component for the rapid activation of cell-associated plasminogen by tissue-type plasminogen activator.

Cancer invasion and metastasis are promoted by proteinases that are activated on the tumor cell surface and in the pericellular spaces. These proteinases digest basement membrane and extracellular matrix components, allowing tumor cell penetration through tissue (1). In breast cancer, cellular expression of the serine proteinase, urokinase plasminogen activator (u-PA),[1] has been correlated with an aggressive, malignant phenotype (2, 3). This may reflect the ability of cell-associated u-PA to activate plasminogen that, in turn, digests glycoprotein components of the extracellular matrix and may activate other proteinases (4, 5). A second plasminogen activator, tissue-type plasminogen activator (t-PA), also binds to cell surfaces and activates plasminogen (6). t-PA may substitute for u-PA in promoting tumor cell invasion (7).

The cellular receptor for u-PA has been cloned and characterized (8, 9). uPA receptor is a glycosylphosphatidylinositol-anchored membrane protein that binds u-PA with moderately high affinity (K_D ~1.0 nM). Cellular binding sites for t-PA and plasminogen have also been identified; however, the biochemical nature of these sites remains more vague. Breast cancer cells *in vitro* bind plasminogen in a specific and saturable manner (10, 11). As is typical of most plasminogen/cellular interactions, the binding affinity is modest (K_D ~1.0 μM); however, the binding capacity is high (B_{max} ~10^6 sites per cell). Occupancy of breast cancer cell plasminogen-binding sites *in vivo* probably depends on the concentration of plasminogen in the surrounding tissue, which may be quite high since the plasma concentration of plasminogen is about 1–2 μM.

Plasminogen is activated more readily by u-PA and t-PA when both enzyme and substrate are bound to the surfaces of certain cell types (6, 12–14). Plasmin that is cell-associated reacts very slowly with α_2-antiplasmin and α_2-macroglobulin, which are rapid inhibitors of solution-phase plasmin (15, 16). Thus, the identification and characterization of major cellular binding sites for plasminogen is important for understanding the role of plasmin in tumor cell invasion and other processes that require cell-surface proteolytic activity.

Plasminogen binding to cellular receptors and other macromolecules, including fibrin and specific extracellular matrix proteins, is mediated by its kringle domains, three of which (K1, K4, and K5) express affinity for lysine (17–19). The highest affinity interaction usually involves K1 and proteins with carboxyl-terminal lysines (19, 20). Several plasminogen receptors have been identified in eukaryotic cells. Annexin II, which was identified as an endothelial cell plasminogen receptor, lacks a carboxyl-terminal lysine in its native form and thus requires proteolytic modification by Lys-specific proteinases in order to acquire plasminogen-binding activity (21, 22). α-Enolase, which was identified as a plasminogen receptor in U937

* This work was supported by Grant DAMD17-94-J-4447 from the Department of the Army Breast Cancer Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: University of Virginia Health Sciences Center, Depts. of Pathology and Biochemistry, Box 214, Charlottesville, VA 22908. Tel.: 804-924-9192; Fax: 804-924-8060; E-mail: SLG2T@VIRGINIA.EDU.

[1] The abbreviations used are: u-PA, urokinase plasminogen activator; t-PA, tissue-type plasminogen activator; CK 8, cytokeratin 8; εACA, ε-amino caproic acid; BSA, bovine serum albumin; CPB, carboxypeptidase B; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RFU, relative fluorescence units; VLK-AMC, D-Val-L-Leu-L-Lys-5-aminomethylcoumarin.

monocytoid cells, contains a carboxyl-terminal lysine in its intact form (20). Both α-enolase and annexin II have been demonstrated on the external surfaces of cultured cells, using antibody-based technologies (20, 21); however, the level of expression of cell-surface α-enolase is sufficient to account for only about 10% of the plasminogen binding capacity of U937 cells (23).

Cytokeratin 8 (CK 8) is an intermediate filament protein that polymerizes with CK 18 to form a component of the cytoskeleton in simple epithelia and many epithelial cell-derived neoplasms. CK 8 interacts extensively with the internal leaflet of the plasma membrane and is, therefore, one of the major components recovered in the plasma membrane fraction when epithelial cells are subjected to subcellular fractionation procedures (10). CK 8 is unique among cytokeratins in that the intact structure includes a carboxyl-terminal lysine. In a recent study, we demonstrated that CK 8 is the primary plasminogen-binding protein in the plasma membrane fraction isolated from hepatocytes (10). We then demonstrated, by immunoelectron microscopy and immunofluorescence microscopy, that CK 8 is present on the external surfaces of intact, unpermeabilized hepatocytes, hepatocellular carcinoma cells, and breast cancer cells, confirming previous studies (24, 25) and identifying CK 8 as a candidate plasminogen receptor.

The purpose of the present study was to determine (i) whether cell-surface CK 8 binds plasminogen; (ii) whether CK 8 contributes substantially to the total plasminogen binding capacity of breast cancer cells; and (iii) whether cell-associated CK 8 promotes plasminogen activation. To accomplish these goals, we prepared a monoclonal antibody (1E8) specific for the carboxyl-terminal 12 amino acids of intact CK 8, which was considered the most likely plasminogen-binding site within the CK 8 structure. We then prepared highly purified Fab fragments of 1E8. This was an important step since the primary structure of all mouse and human IgG isotypes includes carboxyl-terminal Lys residues (26), which, unless exposed to plasma carboxypeptidases, bind plasminogen in solution (27, 28). The results presented here demonstrate that CK 8 is the major plasminogen-binding protein in the plasma membrane fraction of breast cancer cells, the primary protein responsible for the majority of the specific binding of plasminogen to intact breast cancer cells in culture, and a required cellular component for the accelerated activation of cell-associated plasminogen by t-PA.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin and L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) were from Boehringer Mannheim. Aprotinin, chloramine T, ε-aminocaproic acid (εACA), phenylmethylsulfonyl fluoride, and bovine serum albumin (BSA) were from Sigma. Porcine pancreatic carboxypeptidase B (CPB) was from Worthington. $Na^{125}I$ was from Amersham Int. D-Val-L-Leu-L-Lys-5-aminomethylcoumarin (VLK-AMC) was from Enzyme Systems Products (Livermore, CA). IODO-BEADS were from Pierce. Earle's balanced salt solution was from Life Technologies, Inc.

Proteins and Antibodies—[Glu^1]Plasminogen was purified from human plasma by the method of Deutsch and Mertz (29). Single-chain tissue-type plasminogen activator (t-PA) was purchased from American Diagnostica. Monoclonal antibody PCK-26, which recognizes CK 5, CK 6, and CK 8, was from Sigma. Of these cytokeratins, only CK 8 is expressed by breast cancer cells (30).

A polypeptide corresponding to the 12 carboxyl-terminal amino acids of human CK 8, preceded by an amino-terminal cysteine, CKLVSESSDVLPK, was synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry on a Gilson AMS 422 multi-peptide synthesizer and purified by high performance liquid chromatography on a Poros flow-through column (PerSeptive Biosystems, MA). The peptide was cross-linked via its amino-terminal cysteine to maleimide-activated keyhole limpet hemocyanin (Pierce) and used to immunize A/J mice (Jackson Labs, ME). Antibody titers were determined using the same peptide cross-linked to BSA. The mouse with the highest titer was given a final intrasplenic booster. Two days later, spleen cells were harvested and fused with SP2/0 mouse myeloma cells. These cells were selected in HAT-containing medium and cloned.

Eighteen clones generated antibodies that specifically bound CK 8. These antibodies were further screened for ability to detect CK 8 by immunofluorescence microscopy and Western blot analysis. One clone (1E8) was selected for subcloning. Ascites was generated by expanding the subclone of 1E8 (1E8-C6-B4) in CAF1 mice (Jackson Labs, ME), and antibody was purified by Protein A chromatography. Antibody 1E8 isotyped as an IgG_{2b}.

Generation of 1E8 Fab Fragments—Fab fragments of antibody 1E8 were generated using the Immunopure Fab Preparation Kit (Pierce). Briefly, 5 mg of antibody were incubated with 1 ml of papain-agarose for 2 h at 37 °C. Digested antibody was separated from papain-agarose and subjected to protein A-Sepharose chromatography. The flow-through was collected in 1-ml fractions and analyzed by SDS-PAGE. Fractions containing Fab fragments were pooled, aliquoted, and stored at −20 °C until needed. Intact antibody did not contaminate the final Fab preparations, as determined by Coomassie staining of gels that contained up to 20 μg of purified Fab per lane. For some experiments, 1E8 Fab preparations were further processed by incubation with CPB-Sepharose for 12 h at 25 °C, to remove any residual carboxyl-terminal lysine residues (as might have been present due to trace contamination with Fc domain). The CPB-Sepharose was prepared by coupling 5 mg of CPB/1 g of CNBr-activated Sepharose.

Cell Lines and Tissue Culture—All cell lines were obtained from the ATCC (Rockville, MD). The breast cancer cell lines, MCF-7 and MDA-MB-157, and the fibroblast cell line, AKR-2B, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. BT20 breast cancer cells were cultured in RPMI 1640, supplemented as above. Cells were typically passaged by trypsin/EDTA treatment and gentle scraping. In order to minimize the effects of trypsin on cell-surface proteins, cultures were maintained for at least 2 days after passaging before performing experiments.

Subcellular Fractionation—Confluent monolayers of breast cancer cells (3–6 × 10^7 cells) were harvested by gentle scraping into 5 mM EDTA and pelleted at 200 × *g*. Whole cell extracts (W fraction) were obtained by Dounce homogenization in the presence of 10 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μM leupeptin, and 0.2 μg/ml E-64. The W fraction was centrifuged at 250 × *g* for 20 min in an Eppendorf microcentrifuge. The resulting pellet consisted of nuclei and unhomogenized cells (N fraction). The supernatant was centrifuged at 1250 × *g* for 20 min. The resulting pellet was enriched in plasma membrane but partially contaminated with mitochondria, whereas the supernatant consisted of cytoplasm and organelles, including mitochondria (C fraction). The plasma membrane-enriched pellet was resuspended in 1.45 M sucrose (400 μl) and overlaid with 0.25 M sucrose in a 500-μl airfuge tube. The step gradient was centrifuged at 55,000 × *g* in an airfuge for 45 min. Enriched plasma membranes (P fraction) were collected from the interface of the two sucrose solutions (31).

SDS-PAGE, Western Blotting, and Plasminogen Overlay Assays—Subcellular fractions from breast cancer cells were subjected to SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore) using a Hoefer Transphor apparatus (2 h, 0.5 amp). In some studies, the transferred proteins were stained with 0.2% (w/v) Coomassie Blue R-250 (Bio-Rad). In plasminogen overlay experiments, the membranes were blocked with 5% nonfat dried milk, rinsed twice with 20 mM sodium phosphate, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4 (PBS-T), and then incubated with ^{125}I-plasminogen (10 nM) and aprotinin (10 μM), in the presence and absence of 10 mM εACA or a 50-fold molar excess of non-radiolabeled plasminogen. To determine radioligand binding, the blots were rinsed three times for 15 min in PBS-T, dried, and analyzed using a PhosphorImager. For Western blot analyses, blocked membranes were incubated sequentially with a 1:500 dilution of CK 8-specific antibody (PCK-26 or 1E8), horseradish peroxidase-labeled goat anti-mouse monoclonal antibody, and diaminobenzidine.

^{125}I-1E8 Binding to MCF-7 Cells—Antibody 1E8 was radioiodinated using IODO-BEADS (Pierce). The specific activity was 1–2 μCi/μg. Confluent MCF-7 cells were subcultured into 48-well plates and grown for 48 h. Increasing concentrations of ^{125}I-1E8 (0.02–1.0 μM), in Earle's balanced salt solution, 10 mM HEPES, 10 mg/ml BSA, pH 7.4 (EHB), were incubated with the cells for 2 h at 4 °C. The cells were then rinsed three times with EHB and once with PBS. Cell-associated radioactivity was recovered in 1.0% SDS, 0.1 M NaOH and measured in a γ-counter. For concentrations of ^{125}I-1E8 up to 0.2 μM, nonspecific binding was determined by competition with a 50-fold molar excess of non-radiola-

beled 1E8. Nonspecific binding was plotted as a function of ^{125}I-1E8 concentration and extrapolated to higher concentrations by linear regression. Specific ^{125}I-1E8 binding was then determined, at each concentration, by subtracting the nonspecific binding derived from the standard curve. Specific binding isotherms were fit to the equation for a rectangular hyperbola by nonlinear regression and analyzed by Scatchard transformation.

^{125}I-1E8 Binding to Purified CK 8—Rat hepatocyte CK 8 was purified by the method of Achtstaetter *et al.* (32). In the final step of this procedure, CK 8 is eluted from DEAE-Sephacel in a buffer that contains 8.0 M urea. The purified CK 8, at a concentration of 15 μg/ml, was diluted 1:3 with PBS and incubated in 48-well tissue culture plates for 1.5 h at 37 °C. The plates were rinsed three times and blocked with 10 mg/ml BSA for 1.5 h at 37 °C. ^{125}I-1E8 binding was studied using the method described for the cell cultures in the previous section.

^{125}I-Plasminogen Binding to Breast Cancer Cells in the Presence of 1E8 Fab—In order to determine the contribution of cell-surface CK 8 to the total plasminogen binding capacity of breast cancer cells, competition binding experiments were performed. 1E8 Fab (0.5–8.0 μM) was preincubated with MCF-7 cells for 2 h at 4 °C. In some experiments, the cultures were washed three times with EHB to ensure that all unbound 1E8 Fab was removed. In other studies, the 1E8 Fab was allowed to remain in the cultures. ^{125}I-Plasminogen (0.2 μM) was then added in the presence and absence of 10 mM ϵACA or a 50-fold molar excess of unlabeled plasminogen, for 1 h at 4 °C. Unlabeled plasminogen and ϵACA are equally effective at displacing ^{125}I-plasminogen from specific cellular binding sites (12, 13). At the conclusion of an incubation, the cultures were washed three times. Cell-associated radioactivity was recovered in SDS/NaOH and measured in a γ-counter. For the protocol in which unbound 1E8 Fab was not removed by washing prior to adding ^{125}I-plasminogen, the 1E8 Fab was always pretreated with CPB-Sepharose. In control experiments, the effects of monoclonal antibody 1D7 Fab on ^{125}I-plasminogen binding to MCF-7 cells was studied. 1D7 is specific for human α_2-macroglobulin. ^{125}I-Plasminogen binding was also studied in cultures of BT20 cells, MDA-MB-157 cells, and AKR-2B fibroblasts, with and without prior 1E8 Fab treatment. Fibroblasts do not express CK 8 (this was confirmed by Western blotting with PCK-26 and 1E8) and thus cannot bind 1E8 Fab in a specific manner.

Plasminogen Activation by t-PA—Increasing concentrations of plasminogen (0.05–1.0 μM) were incubated with MCF-7 cells or in wells without cells for 1 h at 22 °C. The MCF-7 cell cultures were washed so that only cell-associated plasminogen remained. t-PA (2 nM) and the plasmin-specific fluorescent substrate, VLK-AMC (0.5 mM), were added simultaneously to the MCF-7 cell cultures and to the wells without cells. Plasminogen activity was detected by continuous monitoring (30-s intervals) of fluorescence emission using a Cytofluor 2350 fluorescent plate reader (Millipore). The excitation wavelength was 380 nm, and the emission wavelength was 480 nm (5-nm slit widths). Curves of fluorescence against time were transformed using a first derivative function so that the resulting plots showed the concentration of active plasmin against time. Amounts of plasminogen, which bound to the MCF-7 cells, were determined separately, by performing equivalent incubations with ^{125}I-plasminogen. The velocity of VLK-AMC hydrolysis (0.5 mM) by MCF-7 cell-associated plasmin was decreased by 45 ± 3% (n = 4) compared with solution-phase plasmin; the presented graphs were not corrected for this difference in substrate hydrolysis rate. No VLK-AMC hydrolysis was observed when either plasminogen or t-PA were omitted from the reaction.

To determine whether CK 8 affects the kinetics of plasminogen activation by t-PA on the cell surface, MCF-7 cells were preincubated for 1 h at 22 °C with increasing concentrations of 1E8 Fab (1.0–8.0 μM) or with vehicle. After washing the cultures, 1.0 μM plasminogen was added for 1 h. After washing the cells again, t-PA (2 nM) and VLK-AMC (0.5 mM) were added simultaneously. Plasminogen activation was determined by monitoring fluorescence emission.

Immunofluorescence Microscopy—BT20 and MCF-7 cells were passaged onto 30-mm glass coverslips and grown for at least 48 h. The cells were washed with EHB buffer and incubated with purified 1E8 at 1/200 dilution for 2 h at 4 °C. The cells were then washed three times with EHB, incubated with Texas Red-labeled goat anti-mouse IgG (1/1000 dilution) for 2 h at 4 °C, rinsed three times again, and fixed in ice-cold paraformaldehyde. Cellular immunofluorescence was imaged using an Olympus 3H2 Microscope. In control experiments, mouse nonimmune IgG was substituted for primary antibody or primary antibody was omitted.

FIG. 1. **CK 8 specificity of antibody 1E8.** BT20 whole cell lysate (100 μg) was subjected to Western blot analysis using the well characterized CK 8-specific antibody, PCK-26, and the newly generated antibody, 1E8. Molecular mass standards (in-kDa) are shown.

RESULTS

Characterization of Monoclonal Antibody 1E8—Whole cell extracts from BT20 breast cancer cells were subjected to SDS-PAGE and Western blot analysis using the newly generated monoclonal antibody, 1E8. Only a single protein immunoreacted with 1E8. This protein was CK 8, as determined by its molecular mass (55 kDa) and immunoreactivity with antibody PCK-26 (Fig. 1). In control experiments, identical amounts of protein from whole cell extracts of AKR-2B cells, which lack CK 8, were subjected to Western blot analysis with antibody 1E8. No immunoreactivity was observed (results not shown).

We previously performed indirect immunofluorescence microscopy experiments, using antibodies AB 6.01 and M20, which recognize CK 8, and demonstrated the presence of CK 8 or a CK 8-like protein on the external surfaces of intact, unpermeabilized breast cancer cells (10). To determine whether the carboxyl terminus of CK 8, which we considered the most likely plasminogen-binding site, was exposed on the surfaces of breast cancer cells, immunofluorescence microscopy studies were performed with antibody 1E8. Fig. 2 shows that antibody 1E8 bound to live, nonpermeablized BT20 cells, forming a diffuse and punctate pattern, identical to that seen with other CK8-reactive antibodies (PCK-26, AB 6.01) (10). All of the cells in each preparation showed positive immunofluorescence, irrespective of whether they were in clusters or isolated. When primary antibody was omitted or when mouse nonimmune IgG was substituted for antibody 1E8, no immunostaining was observed. Equivalent results were obtained with MCF-7 cells (results not shown); however, AKR-2B fibroblasts were immunonegative with antibody 1E8, as expected (data not shown).

Plasminogen Overlay Assays—In our previous study (10), we performed plasminogen overlay assays and demonstrated that CK 8 is the major plasminogen-binding protein in the P fraction (plasma membranes) of rat hepatocytes. Fig. 3 shows that similar results were obtained when three separate breast cancer cell lines were analyzed. In the P fraction from each cell line, the major plasminogen-binding protein had an apparent mass of 55 kDa, identical to that of CK 8. The MDA-MB-157 cell P fraction showed a second, plasminogen-binding species, with an apparent mass of 35 kDa. This 35-kDa band was also present as a minor component in the P fraction from the MCF-7 cells. ^{125}I-Plasminogen binding to the 55- and 35-kDa bands was completely inhibited when ϵACA or excess unlabeled plasminogen was added (results not shown).

In the whole cell (W) extract from all three breast cancer cell lines, the 55-kDa band was the major plasminogen-binding

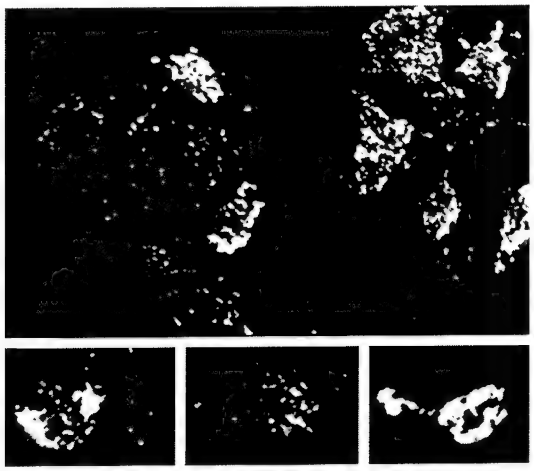


FIG. 2. **Indirect immunofluorescence microscopy of BT20 breast cancer cells.** Viable, nonpermeabilized BT20 breast cancer cells were immunolabeled with monoclonal antibody 1E8, followed by Texas Red-labeled goat anti-mouse IgG. The *top panel* shows a field with two clusters of BT20 cells. The *three lower fields* show isolated cells within the preparation. All cells displayed a similar pattern of diffuse, punctate cell surface immunolabeling.

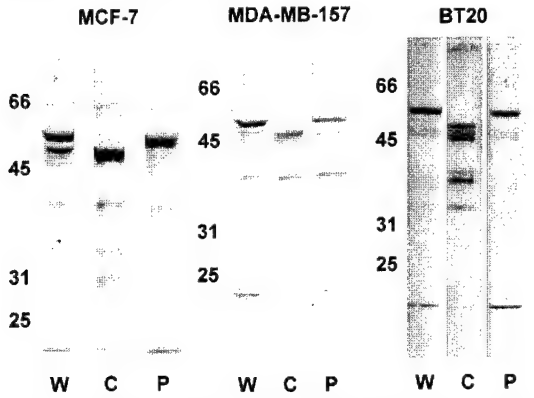


FIG. 3. **^{125}I-Plasminogen overlay assays of breast cancer cell subcellular fractions.** Subcellular fractions (*W*, whole cell extract; *C*, cytoplasm; *P*, plasma membrane) from the three breast cancer cell lines were subjected to SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and incubated with 10 nM ^{125}I-plasminogen. Binding was detected by PhosphorImager analysis. Molecular mass standards (in kDa) are shown.

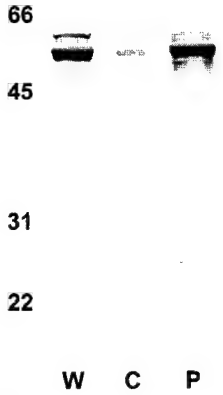


FIG. 4. **Western blot analysis of BT20 subcellular fractions.** The identical BT20 breast cancer cell subcellular fractions, shown in Fig. 3, were subjected to Western blot analysis with antibody PCK-26. Molecular mass standards (in kDa) are shown.

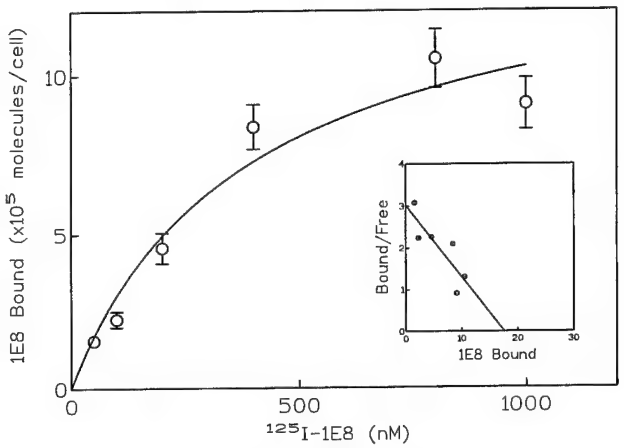


FIG. 5. **Specific binding of ^{125}I-1E8 to MCF-7 breast cancer cells.** Increasing concentrations of ^{125}I-1E8 were incubated with MCF-7 cells in the presence and absence of excess unlabeled 1E8. Specific binding is shown. Each point represents the average ± S.E. of four replicate determinations in a single study, which is representative of three separate experiments. The Scatchard transformation is shown in the *inset*.

component. The prominent appearance of the 55-kDa band in the W fractions distinguished the breast cancer cells from hepatocytes; in the hepatocyte W fraction, CK 8 was a minor plasminogen-binding component (10). The 55-kDa band was excluded from the breast cancer cell cytoplasmic fractions (C), as would be expected for CK 8.

To confirm that the 55-kDa band observed in the plasminogen overlay assays was CK 8, the identical BT20 subcellular fractions were subjected to Western blot analysis with antibody PCK-26. The antibody detected large amounts of CK 8 in both the W and P fractions, while a greatly reduced level of antigen was detected in the C fraction (Fig. 4). The mobility of the major band detected in the Western blot analysis coincided exactly with the mobility of the 55-kDa band in the plasminogen overlay assays.

Specific Binding of Antibody 1E8 to MCF-7 Cells—Radioiodinated antibody 1E8 bound to MCF-7 cells in a specific, saturable manner. A representative binding isotherm is shown in Fig. 5. An unexpected finding in this and two other equivalent studies was that the results transformed into apparently linear Scatchard plots. While not conclusive, this result suggests that each antibody engages only a single CK 8 epitope. The K_D for 1E8 binding to MCF-7 cells was 0.4 ± 0.1 μM and the B_{max} was 1.5 ± 0.5 × 10^6 sites per cell (n = 3). The B_{max} for 1E8 is comparable with that determined for plasminogen binding to the same cell type (10).

In control experiments, binding of intact ^{125}I-1E8 to purified, immobilized CK 8 was studied. ^{125}I-1E8 bound specifically and saturably to the CK 8, whereas no binding was observed in BSA-coated wells in two separate experiments. The data transformed into linear Scatchard plots ($r > 0.95$) and a K_D of 0.24 μM was derived (results not shown).

1E8 Fab Fragments Block Plasminogen Binding to Breast Cancer Cell Lines—The contribution of cell-surface CK 8 to the plasminogen binding capacity of breast cancer cells was studied using 1E8 Fab and MCF-7 cells, as a representative cell line. When 1E8 Fab was coincubated with ^{125}I-plasminogen in the MCF-7 cell cultures, specific radioligand binding was decreased and the extent of the decrease was 1E8 Fab concentration-dependent (Fig. 6). With the highest concentration of 1E8 Fab (8 μM), specific plasminogen binding was decreased by 82%.

In separate experiments, MCF-7 cells were preincubated with 1E8 Fab and then washed to remove unbound Fab prior to adding ^{125}I-plasminogen. It was anticipated that this protocol would decrease the level of observed competition since 1E8 Fab dissociation from the cell surface would be favored while the ^{125}I-plasminogen is present in the cultures. However, as shown

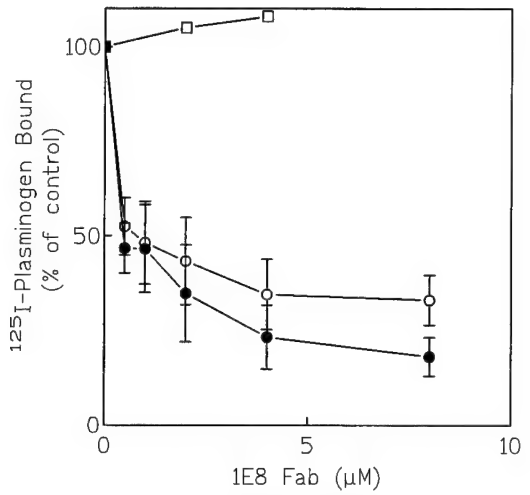


FIG. 6. **1E8 Fab inhibits ^{125}I-plasminogen binding to MCF-7 cells.** 1E8 Fab was preincubated with MCF-7 or AKR-2B cells for 2 h at 4 °C. In the first protocol, ^{125}I-plasminogen was added to the MCF-7 cell cultures without first washing the cells so that unbound 1E8 Fab remained (●). In the second protocol, the MCF-7 cells were washed to remove unbound 1E8 Fab prior to adding ^{125}I-plasminogen (○). The AKR-2B cells were washed after incubation with 1E8 Fab and prior to adding the ^{125}I-plasminogen (□). Specific plasminogen binding is expressed as a percentage of that observed without 1E8 Fab treatment. The presented results, for the MCF-7 cells, show the mean ± S.E. for five separate experiments.

in Fig. 6, significant inhibition of specific ^{125}I-plasminogen binding was still observed. In cells that were pretreated with 8 μM 1E8 Fab, ^{125}I-plasminogen binding was decreased by 67%. In control experiments, no decrease in ^{125}I-plasminogen binding was observed when AKR-2B fibroblasts were pretreated with 1E8 Fab. Antibody 1D7 Fab had no effect on ^{125}I-plasminogen binding to MCF-7 cells, irrespective of whether the Fab was present during the incubation with plasminogen or washed-out prior to adding plasminogen (results not shown). The ability of 1E8 Fab to inhibit plasminogen binding to MCF-7 cells, even after the free Fab was removed by washing, confirms that the observed competition is due to blocking of plasminogen-binding sites on the cell surface and not a solution-phase interaction with ^{125}I-plasminogen.

Binding of ^{125}I-plasminogen to BT20 and MDA-MB-157 cells was studied after pretreating the cultures with 4 μM 1E8 Fab. The cells were washed prior to adding ^{125}I-plasminogen to remove unbound 1E8 Fab. As shown in Table I, specific ^{125}I-plasminogen binding to the BT20 cells was decreased, and the magnitude of the effect was only slightly less than that observed with MCF-7 cells. Decreased plasminogen binding was also observed with the MDA-MB-157 cells; however, the effectiveness of 1E8 Fab was not as great in this cell line. In control experiments, we studied the effects of 1E8 Fab on plasminogen binding to purified, immobilized CK 8. 1E8 Fab (4 μM) was incubated with the CK 8. The wells were then washed before adding ^{125}I-plasminogen. In two separate experiments, specific ^{125}I-plasminogen binding was decreased by 62 and 68%. Thus, the extent of competition observed in the BT20 and MCF-7 cell cultures was similar to that detected using the identical protocol, in a purified system that includes CK 8 as the exclusive plasminogen-binding protein.

MCF-7 Cells Enhance Plasminogen Activation by t-PA—To study plasminogen activation by t-PA, increasing concentrations of plasminogen were added to MCF-7 cell cultures and to wells without cells. The cell cultures were washed, so that only cell-associated plasminogen remained (less than 3% of the plasminogen originally added to the wells). The wells without cells were not washed. t-PA and VLK-AMC were then added simultaneously to each well. In the MCF-7 cell cultures, the velocities of plasminogen activation showed an initial lag phase lasting about 2 min, followed by a 5–7-min period when the velocities optimized and the first derivative functions (dRFU/dt) linearized. RFU indicates relative fluorescence units. The period between 2.5 and 7 min was used to derive the plasminogen activation rates shown in Fig. 7 (*panel A*).

TABLE I
Inhibition of ^{125}I-plasminogen binding to breast cancer cell lines by 1E8 Fab

All cultures were pretreated with 4 μM 1E8 Fab and then washed before adding plasminogen. The column labeled CK 8 shows the inhibition observed in wells coated with purified CK 8 (no cells). All values represent the mean ± S.E.

	BT20 (n = 3)	MDA-MB-157 (n = 3)	MCF-7 (n = 6)	CK 8 (n = 2)
% inhibition	53 ± 3	39 ± 3	64 ± 8	65 ± 3

In wells without cells, plasminogen activation proceeded without an apparent lag phase. Rates of plasminogen activation (determined in the 2.5–7-min time interval) were slightly decreased compared with the rates measured in cell cultures that were initially incubated with the identical concentrations of plasminogen. However, since the wells without cells were not washed before adding t-PA, the amount of available plasminogen was actually about 100-fold greater. Fig. 7, *panel B*, shows Lineweaver-Burk transformations of the activation data. In the transformations, the actual amount of available substrate (plasminogen) is plotted as opposed to the amount initially added to the wells. Levels of cell-associated plasminogen are converted into units of concentration by dividing the number of moles of bound plasminogen by the total incubation volume (100 μl). The most remarkable finding was a 2000-fold reduction in the K_m for the activation of cell-associated plasminogen (2 nM) compared with plasminogen in solution (4 μM). Although the K_m for plasminogen activation in solution is similar to previously reported constants (33, 34), this value should be interpreted cautiously since the plasminogen concentration range was selected to match the cell culture studies and is substantially lower than that required for optimal accuracy. The V_{max} for plasminogen activation by t-PA was decreased about 3-fold when the plasminogen was cell-associated.

In order to study the role of cell-surface CK 8 in promoting the activation of plasminogen by t-PA, MCF-7 cells were pretreated with increasing concentrations of 1E8 Fab. The cells were then washed and incubated with plasminogen. After a final wash, t-PA and VLK-AMC were added simultaneously, and fluorescence was monitored. In this assay, some 1E8 Fab probably dissociated from the cell surfaces during the 1-h incubation with plasminogen at 22 °C. Nevertheless, as shown in Fig. 8, preincubation with 1E8 Fab caused a concentration-dependent decrease in the ability of the cell cultures to promote plasminogen activation. After incubation with 8 μM 1E8 Fab, the velocity of plasminogen activation was decreased by 83 ± 6%.

DISCUSSION

Numerous studies have demonstrated that cell-associated plasmin and plasminogen activators promote tumor cell invasion in *in vitro* model systems (1, 35, 36). An increasing number of studies support the function of these same proteinases and their cellular receptors in tumor invasion and metastasis *in vivo*. For example, Mueller *et al.* (37) demonstrated that melanoma cells, when transfected to overexpress plasminogen activator inhibitor 2, form subcutaneous tumors that do not metastasize in *scid* mice, unlike the highly metastatic parent cell line. Evans and Lin (38) blocked implantation of intravenously

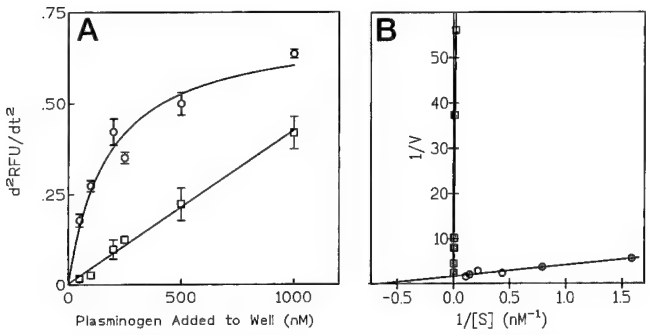


FIG. 7. **Activation of MCF-7 cell-associated plasminogen by t-PA.** *Panel A* shows rates of activation of MCF-7 cell-associated plasminogen (○) and plasminogen in solution (□) by 2 nM t-PA (mean ± S.E., $n = 4$). Plasminogen activation was detected by monitoring VLK-AMC hydrolysis. Activation rates were calculated from second derivatives (d^2RFU/dt^2) of the plots of fluorescence against time, beginning 2.5 min after adding the VLK-AMC. In *panel A*, the listed concentrations of plasminogen are the amounts that were added to the wells. In the cell cultures, the majority of the plasminogen was removed by washing before adding t-PA. *Panel B* shows Lineweaver-Burk transformations of the results presented in *panel A*. In *panel B*, the actual amount of plasminogen present in the well when the t-PA and VLK-AMC were added is shown.

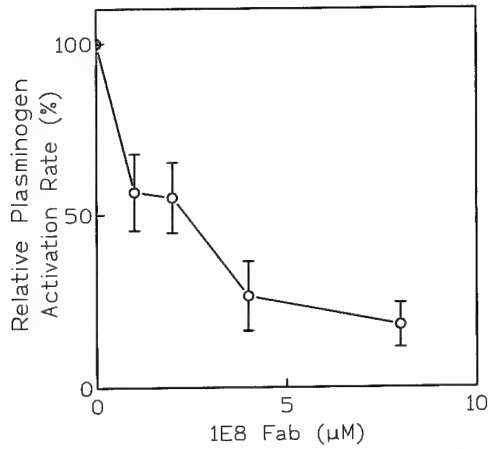


FIG. 8. **Effects of 1E8 Fab on the activation of MCF-7 cell-associated plasminogen.** 1E8 Fab was incubated with MCF-7 cells for 1 h at 22 °C. The cultures were washed, and plasminogen (1.0 μM) was added for 1 h. After washing again, t-PA and VLK-AMC were added, and fluorescence emission was monitored (mean ± S.E., $n = 3$). The relative plasminogen activation rate is plotted as a percentage of that observed with cells that were not 1E8 Fab-treated.

administered breast cancer cells, in the lungs of rats, by co-administering recombinant plasminogen activator inhibitor 2. Finally, Kobayashi *et al.* (39) demonstrated that urinary trypsin inhibitor, which neutralizes cell-associated plasmin, also inhibits cancer cell invasion through Matrigel *in vitro* and blocks formation of lung metastases by Lewis lung carcinoma cells *in vivo*. By contrast, the potent solution-phase plasmin inhibitors, α_2-antiplasmin and α_2-macroglobulin, which are ineffective against cell-associated plasmin, show no activity in the same cancer cell invasion assays (39). These studies emphasize the importance of identifying macromolecules responsible for plasminogen binding in cancer.

Our initial studies with rat hepatocytes identified CK 8 as a potential plasminogen receptor in epithelial cells (10). Since prior evidence for the presence of CK 8 on the outer surfaces of cells had been disputed, immunofluorescence and immunoelectron microscopy studies were performed. The fluorescence microscopy studies of hepatocytes and breast cancer cell lines showed that CK 8 is distributed uniformly on the surfaces of viable, adherent cells and not restricted to a fraction of each population, such as injured cells (10). We confirmed that the cell preparations were, in fact, unpermeabilized and that the CK 8-specific antibodies recognized cell-surface CK 8 and not intracytoplasmic CK 8 by electron microscopy. However, since the epitopes recognized by commercially available CK 8-specific antibodies are uncharacterized, it was not possible to conclude that the region of CK 8 responsible for plasminogen binding is exposed on the cell surface. Thus, monoclonal antibody 1E8 was raised utilizing synthetic peptide technology. The similarity in the distribution of 1E8 immunofluorescence, compared with previously studied CK 8-specific antibodies (10), suggests that the carboxyl terminus of CK 8 is intact and available for protein-binding interactions on breast cancer cell surfaces. Furthermore, our binding studies with ^{125}I-1E8 provide an estimate of the amount of CK 8 on the outer surfaces of MCF-7 cells (1–2 million sites per cell). The density of CK 8 on the MCF-7 cell surface is sufficient to account for the high cellular binding capacity for plasminogen (10).

Competition binding experiments with ^{125}I-plasminogen and 1E8 were performed using two separate methods. The methods were designed to ensure that activity resulted from antibody binding to cell-surface CK 8 and not interaction with ^{125}I-plasminogen in solution. In early studies, we routinely screened antibody preparations for plasminogen-binding activity in solution by testing the ability of each antibody to inhibit ^{125}I-plasminogen binding to Lys-Sepharose. Various CK 8-specific monoclonal antibodies (1E8, PCK-26, AB 6.01, M20) bound plasminogen in solution, as determined by the Lys-Sepharose competition assay. 1E8 Fab preparations demonstrated little or no plasminogen binding activity, and after treatment with CPB-Sepharose, were entirely inactive. The variable amount of residual plasminogen binding activity in Fab preparations, without CPB-Sepharose treatment, may have reflected trace-level contamination with Fc domain or perhaps proteolytic modification of the Fab itself. When cell cultures were pretreated with 1E8 Fab and then washed to remove free 1E8 Fab, before adding ^{125}I-plasminogen, any residual plasminogen binding activity that may have been associated with the Fab could only have increased the level of observed plasminogen binding to the cells. Thus, the 1E8 Fab "wash-out" protocol provided the most rigorous demonstration of receptor competition even though this method probably provided a minimum estimate of the contribution of CK 8 to the plasminogen binding capacity, due to 1E8 Fab dissociation from the cell surface during the incubation with plasminogen.

Of the three breast cancer cell lines studied, the greatest effects of 1E8 Fab on plasminogen binding were observed with MCF-7 cells. When CPB-Sepharose-treated 1E8 Fab was retained in the culture medium during the incubation with ^{125}I-plasminogen, binding was inhibited by up to 82%. Plasminogen binding was inhibited by 60–70% when the free 1E8 Fab was removed prior to adding ^{125}I-plasminogen. The difference between these two values suggests that 1E8 Fab may have partially dissociated from the cell surface after Fab washout but that substantial levels of cell-associated 1E8 Fab remained. In our studies comparing cell lines, 10% less competition was observed with BT20 cells and 25% less competition was observed with MDA-MB-157 cells, compared with the MCF-7 cells; however, at least with the BT20 cells, CK 8 still accounted for more than one-half of the plasminogen-binding sites. Since the Fab washout protocol was used in the experiments with MDA-MB-157 cells, it is quite possible that CK 8 accounts for more than 50% of the plasminogen-binding sites in this cell line as well. Thus, we conclude that CK 8 is a major plasminogen-binding protein in various breast cancer cell lines and may be

responsible for the great majority of the plasminogen-binding sites in some.

To demonstrate maximum inhibition of ^{125}I-plasminogen binding, we used high concentrations of 1E8 Fab; however, we still may not have entirely saturated available CK 8 binding sites for plasminogen. If the affinity of 1E8 Fab and intact antibody for CK 8 are equivalent (~0.4 μM), then at equilibrium 1E8 Fab at 4 and 8 μM would be expected to occupy 91 and 95% of the available epitopes, respectively. If the affinity of 1E8 Fab for cell-surface CK 8 was reduced compared with intact antibody, then the degree of saturation would be less. In any case, the unoccupied CK 8 may be responsible for a significant fraction of the residual plasminogen binding, especially in experiments with the MCF-7 cells that were the most profoundly affected by 1E8 Fab.

Even though there are data to support an important role for u-PA in determining the metastatic potential of breast cancer cells (2, 3), we chose to assess the functional significance of plasminogen/breast cancer cell interactions by examining the rate of plasminogen activation by t-PA. We and others (6, 12, 13) have shown that only certain cell types, including hepatocytes, promote plasminogen activation by t-PA. We hypothesized that cell-surface CK 8 might be responsible for the previously demonstrated enhancement of plasminogen activation by t-PA in hepatocytes (12) and that CK 8 might promote plasminogen activation by t-PA in breast cancer cells. Our analysis of MCF-7 cells demonstrated that plasminogen activation by t-PA is greatly accelerated when the plasminogen is cell-associated. The increase in activation rate was attributed to a substantial decrease in K_m. From the data presented, it was not possible to conclude whether the enhanced rate of plasminogen activation requires only plasminogen binding to CK 8 or a concomitant interaction of t-PA with CK 8 or another surface binding site. In any respect, the 83% decrease in plasminogen activation rate after 1E8 Fab pretreatment demonstrates that CK 8 plays an essential role in promoting the activation of plasminogen by t-PA on the MCF-7 cell surface. The lag phase observed in the analysis of cell-associated plasminogen activation by t-PA provides suggestive evidence that t-PA may need to bind to a cell-surface site before it acquires optimal catalytic efficiency. If this is true, then one could postulate a model for enhanced plasminogen activation by CK 8 that is similar to that originally proposed for fibrin (40). In this model CK 8 would mediate the formation of a ternary complex in which the K_m for plasminogen activation is greatly decreased. In studies with purified CK 8, we recently demonstrated specific t-PA binding (41).

CK 8 can be aberrantly expressed in many nonepithelial cancers, including lymphomas, melanomas, gliomas, and sarcomas (42, 43). For many of these cancers, expression of CK 8 has been correlated with increased invasiveness *in vitro* and *in vivo*. When CK 8 is aberrantly expressed in squamous cell carcinomas, it is localized, by immunohistochemistry, primarily to the invasion front (44, 45). In malignant melanoma, *in vitro* invasiveness has been directly correlated with cellular expression of CK 8 (46). Finally, mouse L cells, a noninvasive CK 8-negative fibroblast cell line, becomes invasive after cotransfection with CK 8 and its co-polymer CK 18 (47). Based upon our work, we hypothesize that the increased invasiveness of CK 8-expressing cells may be partially explained by the small fraction of CK 8 that is available at the cell surface and competent to function as a plasminogen receptor.

The mechanism by which CK 8 is expressed on the cell surface is unknown. The sequence of CK 8 does not include a transmembrane domain; however, our unpublished preliminary data[2] suggest that cell-surface CK 8 is integrally associated with the plasma membrane. The alternate hypothesis is that CK 8, which is released by cells, binds to the external plasma membrane and then functions as an extrinsic receptor for plasminogen. In either case, the functional significance of CK 8, which is its ability to bind plasminogen and promote plasminogen activation on the tumor cell surface, is the same. We propose that cell-surface CK 8 may be important in promoting invasion of breast cancer cells.

Acknowledgments—We thank Dr. William Sutherland, Deborah Koons, and Bucky Bailes of the Lymphocyte Culture Center at the University of Virginia for assistance in preparing monoclonal antibody 1E8 and in generating AB 6.01 ascites. We also thank Kristine Garza for synthesizing the CK 8 carboxyl-terminal peptide.

REFERENCES

1. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. (1991) *Cell* **64,** 327–336
2. Duffy, M. J., Reilly, D., O'Sullivan, C., O'Higgens, N., Fennelly, J. J., and Andreasen, P. (1990) *Cancer Res.* **50,** 6827–6829
3. Bianchi, E., Cohen, R. L., Thor, A. T., Todd, R. L., Mizukami, I. F., Lawrence, D. A., Ljung, B. M., Shuman, M. A., and Smith, H. S. (1994) *Cancer Res.* **54,** 861–866
4. Liotta, L. A., Goldfarb, R. H., Brundage, R., Siegal, G. P., Teranova, V., and Gabrisa, S. (1981) *Cancer Res.* **41,** 4629–4635
5. Mignatti, P., Robbins, E., and Rifkin, D. B. (1986) *Cell* **47,** 487–498
6. Hajjar, K. A., Hamel, N. M., Harpel, P. C., and Nachman, R. L. (1987) *J. Clin. Invest.* **80,** 1712–1719
7. Alizadeh, H., Ma, D., Berman, M., Bellingham, D., Comerford, S. A., Gething, M. J. H., Sambrook, J. F., and Niederkorn, J. Y. (1995) *Curr. Eye Res.* **14,** 449–458
8. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Dano, K., Apella, E., and Blasi, F. (1990) *EMBO J.* **9,** 467–474
9. Behrendt, N., Ronne, E., Ploug, M., Petri, T., Lober, D., Nielsen, L. S., Schleuning, W.-D., Blasi, F., Appella, E., and Dano, K. (1990) *J. Biol. Chem.* **265,** 6453–6460
10. Hembrough, T. A., Vasudevan, J., Allietta, M. M., Glass, W. F., and Gonias, S. L. (1995) *J. Cell Sci.* **108,** 1071–1082
11. Correc, P., Fondaneche, M. C., Bracke, M., and Burtin, P. (1990) *Int. J. Cancer* **46,** 745–750
12. Hajjar, K. A., Harpel, P. C., Jaffe, E. A., and Nachman, R. L. (1986) *J. Biol. Chem.* **261,** 11656–11662
13. Hall, S. W., VandenBerg, S. R., and Gonias, S. L. (1990) *J. Cell. Biochem.* **43,** 213–227
14. Ellis, V., Behrendt, N., and Dano, K. (1991) *J. Biol. Chem.* **266,** 12752–12758
15. Plow, E. F., Herren, T., Redlitz, A., Miles, L. A., and Hoover-Plow, J. L. (1995) *FASEB J.* **9,** 939–945
16. Gonias, S. L. (1992) *Exp. Hematol.* **20,** 302–311
17. Fleury, V., and Angles-Cano, E. (1991) *Biochemistry* **30,** 7630–7638
18. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., and Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis* **3,** 191–209
19. Christensen, U. (1985) *FEBS Lett.* **182,** 43–46
20. Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. (1991) *Biochemistry* **30,** 1682–1691
21. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) *J. Biol. Chem.* **269,** 21191–21197
22. Cesarman, G. M., Guevera, C. A., and Hajjar, K. A. (1994) *J. Biol. Chem.* **269,** 21198–21203
23. Redlitz, A., Fowler, B. J., Plow, E. F., and Miles, L. A. (1995) *Eur. J. Biochem.* **227,** 407–415
24. Godfroid, E., Geuskens, M., Dupressoir, T., Parent, I., and Szpirer, C. (1991) *J. Cell Sci.* **99,** 595–607
25. Chan, R., Rossitto, P. V., Edwards, B. F., and Cardiff, R. D. (1985) in *Monoclonal Antibodies and Breast Cancer* (Ceriani, R. L., ed) pp. 165–178, Nijhoff, Boston
26. Burton, D. R. (1987) in *Molecular Genetics of Immunoglobulins* (Calabi, F., and Neuberger, M. S., eds) pp. 1–47, Elsevier Science Publishing Co., Inc., New York
27. Ellis, V., and Dano, K. (1993) *J. Biol. Chem.* **268,** 4806–4813
28. Redlitz, A., Tan, A. K., Eaton, D. L., and Plow, E. F. (1995) *J. Clin. Invest.* **96,** 2534–2538
29. Deutsch, D. G., and Mertz, E. T. (1970) *Science* **170,** 1095–1096
30. Moll, R., Franke, W. W., and Schiller, D. L. (1982) *Cell* **31,** 11–24
31. Fleischer, S., and Kervina, M. (1986) *Methods Enzymol.* **31,** 6–41
32. Achtstaetter, T., Hatzfeld, M., Quinlan, R. A., Parmelee, D. C., and Franke, W. W. (1986) *Methods Enzymol.* **134,** 355–371
33. Urano, T., Sator de Serrano, V., Gaffney, P. J., and Castellino, F. J. (1988) *Biochemistry* **27,** 6522–6528
34. Liu, J., and Gurewich, V. (1993) *J. Biol. Chem.* **268,** 12257–12259
35. Mignatti, P., and Rifkin, D. B. (1993) *Physiol. Rev.* **73,** 161–201
36. Ellis, V., and Dano, K. (1992) *Fibrinolysis* **6,** (suppl.) 27–34
37. Mueller, B. M., Yu, Y. B., and Laug, W. E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 205–209
38. Evans, D. M., and Lin, P. L. (1995) *Am. Surg.* **61,** 692–697
39. Kobayashi, H., Shinohara, H., Takeuchi, K., Itoh, M., Fujie, M., Saitoh, M., and

[2] T. A. Hembrough, L. Li, and S. L. Gonias, unpublished data.

Terao, T. (1994) *Cancer Res.* **54,** 844–849
40. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) *J. Biol. Chem.* **257,** 2912–2919
41. Hembrough, T. A., Kralovich, K. R., Li, L., and Gonias, S. L. (1996) *Biochem. J.* **317,** 763–769
42. Meittinen, M., and Franssila, K. (1989) *Lab. Invest.* **61,** 62–628
43. Azumi, N., and Battifora, H. (1987) *Am. J. Clin. Pathol.* **88,** 286–296
44. Schaafsma, H. E., Ramaekers, F. C. S., van Muijin, G. N. P., Robben, H., Lane, E. B., Leigh, I. M., Ooms, E. C. M., Schalken, J. A., van Moorselaar, R. J. A., and Ruiter, D. J. (1991) *Am. J. Pathol.* **139,** 1389–1400
45. Schaafsma, H. E., van der Velden, L. A., Manni, J. J., Peters, H., Link, M., Ruiter, D. J., and Ramaekers, F. C. S. (1993) *J. Pathol.* **170,** 77–86
46. Hendrix, M. J. C., Seftor, E. A., Chu, Y.-W., Seftor, R. E. B., Nagle, R. B., McDaniel, K. M., Leong, S. P. L., Yohem, K. H., Leibovitz, A. M., Meyskens, F. L., Conaway, D. H., Welch, D. R., Liotta, L. A., and Stetler-Stevenson, W. (1993) *J. Natl. Cancer Inst.* **84,** 165–174
47. Chu, Y.-W., Runyan, R. B., Oshima, R. G., and Hendrix, M. J. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4261–4265

Biochem. J. (1996) **317**, 763–769 (Printed in Great Britain)

Cytokeratin 8 released by breast carcinoma cells *in vitro* binds plasminogen and tissue-type plasminogen activator and promotes plasminogen activation

Todd A. HEMBROUGH*, Kristen R. KRALOVICH*, Li LI† and Steven L. GONIAS*†‡

Departments of *Biochemistry and †Pathology, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

Cell-surface activation of plasminogen may be important in diseases that involve cellular migration, including atherosclerosis and tumour invasion/metastasis. Cytokeratin 8 (CK 8) has been identified as a plasminogen-binding protein expressed on the external surfaces of hepatocytes and breast carcinoma cells [Hembrough, Vasudevan, Allietta, Glass and Gonias (1995) J. Cell Sci. **108**, 1071–1082]. In this investigation, we demonstrate that a soluble form of CK 8 is released into the culture medium of breast cancer cell lines. The released CK 8 is in the form of variably sized polymers that bind plasminogen and promote the activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen by single-chain tissue-type plasminogen activator (sct-PA). To assess the mechanism by which CK 8 promotes plasminogen activation, CK 8 was purified from rat hepatocytes and immobilized in microtitre plates. Immobilized CK 8 bound ^{125}I-plasminogen and ^{125}I-sct-PA in a specific and saturable manner. The K_Ds were 160 ± 40 nM and 250 ± 48 nM, respectively. Activation of plasminogen bound to immobilized CK 8 was accelerated compared with plasminogen in solution, as determined using a coupled-substrate fluorescence assay and SDS/PAGE. The ability of CK 8 to promote plasminogen activation may be important in the pericellular spaces surrounding breast cancer cells and at the cell surface.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is one of the two major plasminogen activators *in vivo*; however, in the absence of protein cofactors, the efficiency of plasminogen activation by t-PA is poor. Proteins that bind t-PA and/or plasminogen have been identified in the plasma and basement membranes [1,2]. The most important of these, from the standpoint of haemostasis, is fibrin, which substantially increases the catalytic efficiency of plasminogen activation [3].

Many cells in culture express specific and saturable binding sites for t-PA and plasminogen. The affinity of cellular receptors for plasminogen is typically low; however, the total cellular plasminogen binding capacity is high (10^5–10^7 sites per cell) [4]. Binding of plasminogen to certain cells, including endothelial cells [5] and hepatocytes [6], promotes activation by t-PA. Like other plasminogen-binding interactions, those involving cellular receptors utilize the five tandem plasminogen kringle domains, three of which (K1, K4 and K5) demonstrate affinity for lysine residues in other proteins [1,2]. The kringle-1 domain contains the highest-affinity lysine-binding site and interacts preferentially with C-terminal lysines [7–10]. Thus, plasma membrane proteins with extracellular C-terminal lysines are most likely to function as plasminogen receptors.

Several cellular proteins have been identified as candidate plasminogen receptors, including α-enolase, on monocytoid cells, and annexin II, on endothelial cells [10,11]. Although these proteins are primarily intracellular in location, each has been identified on the external cell surface, using immunofluorescent antibody-based techniques [10,11]. The structure of α-enolase includes a C-terminal lysine [10]; annexin II does not have a C-terminal lysine and is thus dependent on modification by plasmin or another lysine-specific proteinase in order to acquire plasminogen-binding activity [11].

Recently, we identified cytokeratin 8 (CK 8) as a candidate epithelial cell plasminogen receptor [12]. CK 8 is an intermediate filament protein [13]; however, using fluorescence and immunoelectron microscopy, we demonstrated CK 8 or a CK 8-like protein on the external surfaces of hepatocytes, breast carcinoma cells and hepatocellular carcinoma cells. CK 8 contains a C-terminal lysine residue [14]. Thus, CK 8 has intrinsic plasminogen binding capacity and does not require prior proteolytic modification in order to bind plasminogen.

The interaction of plasminogen with CK 8 may not be restricted to the cell surface since cytokeratins are released in significant quantities by cancer cells *in vitro* and by carcinomas *in vivo*. CK 8 has been identified as a major component of Tissue Polypeptide Antigen, a tumour-associated marker, found in the serum and body fluids of cancer patients [15,16]. Levels of Tissue Polypeptide Antigen have been used to monitor the progression of malignancies [17]. CK 8 is also released into the culture medium of breast cancer cells *in vitro* [18,19]. Whether the released forms of CK 8 retain the ability to bind plasminogen is unknown.

In this investigation, we confirm the results of previous studies [18] by demonstrating the presence of CK 8 in conditioned medium of breast cancer cells *in vitro*. We then demonstrate, for the first time, that the soluble form of CK 8, recovered in cancer

Abbreviations used: t-PA, tissue-type plasminogen activator; sct-PA, single-chain tissue plasminogen activator; CK 8, cytokeratin 8; [Glu^1]plasminogen, native plasminogen; [Lys^{78}]plasminogen, proteolytically modified plasminogen with Lys^{78} as its N-terminus; EBSS, Earle's balanced salt solution; EHB, EBSS, 10 mM Hepes, 10 mg/ml BSA; S-2251, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; S-2288, D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide; VLK-AMC, D-valyl-L-leucyl-L-lysine-5-aminomethylcoumarin; PPACK, phenylprolylarginylchloromethane; BCA, bicinchoninic acid; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanido)butane; ϵACA, ϵ-aminohexanoic acid; u-PA, urokinase; tcu-PA, two-chain urokinase; DTT, dithiothreitol.

‡ To whom correspondence should be addressed.

cell-conditioned medium, retains plasminogen-binding activity. Purified CK 8 also binds t-PA and enhances the efficiency of plasminogen activation by t-PA. Thus, soluble CK 8, released from cancer cells *in vivo*, may promote plasminogen activation in malignancy.

MATERIALS AND METHODS

Materials

D-Valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) and D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288) were from Kabi Vitrum (Stockholm, Sweden). D-Valyl-L-leucyl-L-lysine-5-aminomethylcoumarin (VLK-AMC) was from Enzyme Systems Products (Livermore, CA, U.S.A.). Phenylprolylarginylchloromethane (PPACK) was from Calbiochem (San Diego, CA, U.S.A.). Leupeptin and L-*trans*-epoxysuccinyl-leucylamido(4-guanido)butane (E-64) were from Boehringer Mannheim. Aprotinin, chloramine T, ϵ-aminohexanoic acid (ϵACA), PMSF, BSA and bicinchoninic acid solution (BCA) were from Sigma Chemicals (St. Louis, MO, U.S.A.). $Na^{125}I$ was from Amersham International (Arlington Heights, IL, U.S.A.). Iodobeads were purchased from Pierce (Rockford, IL, U.S.A.). Earle's balanced salt solution (EBSS) was from Gibco Laboratories (St. Lawrence, MA, U.S.A.). Cell culture plasticware was from Costar (Cambridge, MA, U.S.A.).

Proteins and antibodies

[Glu^1]Plasminogen (native plasminogen) was purified from human plasma by the method of Deutsch and Mertz [20]. [Glu^1]Plasminogen was converted into [Lys^{78}]plasminogen (proteolytically modified plasminogen with Lys^{78} as its N-terminus) by incubation with plasmin–Sepharose. Single-chain t-PA (sct-PA) was from American Diagnostica. The concentration of active sct-PA was determined by the velocity of S-2288 hydrolysis, using the kinetic constants ($K_m \sim 1.0$ mM, $k_{cat} \sim 26\ s^{-1}$) provided by the manufacturer. Recombinant single-chain urokinase (u-PA) was generously provided by Dr. Jack Henkin (Abbott Laboratories) and converted into two-chain urokinase (tcu-PA) by incubation with plasmin–Sepharose. Monoclonal antibody PCK-26, which recognizes CK 5, CK 6 and CK 8, was from Sigma. Of these three cytokeratins, only CK 8 is expressed by breast carcinoma cells [13].

Preparation of BT20 cell-conditioned medium

BT20 breast carcinoma cells were allowed to reach 60–75% confluency and then cultured in serum-free medium (RPMI 1640 with 100 units/ml penicillin and 100 μg/ml streptomycin) for 4 days. The conditioned medium was collected and centrifuged at 1000 *g* for 10 min to pellet cellular debris and then concentrated 10-fold using an Amicon concentrator with a YM-10 membrane.

Concentrated BT20 cell-conditioned medium (0.5 ml) was subjected to molecular exclusion chromatography on a Superose 12 column, using a flow rate of 0.4 ml/min. Elution of proteins was detected by constant monitoring of absorbance at 280 nm. Fractions were collected at 1 min intervals. CK 8 was detected in chromatography fractions by immunoblot analysis using antibody PCK-26. In order to calibrate the column, the following proteins were subjected to chromatography under identical conditions: α_2-macroglobulin (720 kDa), mouse IgG (150 kDa), BSA (66 kDa) and cytochrome *c* (14 kDa).

Effects of BT20 cell-conditioned medium on plasminogen activation by t-PA

Activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen (0.05–1.0 μM) by sct-PA (2 nM), in the presence and absence of BT20 cell-conditioned medium, was studied using a continuous assay and the plasmin-specific substrate, S-2251 (0.5 mM). The BT20 cell-conditioned medium was preincubated with plasminogen in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer for 30 min at 22 °C. sct-PA and S-2251 were added to start the reaction. In the final incubation mixture, the BT20 cell-conditioned medium was diluted 1:2. Absorbance at 406 nm was measured at 5 s intervals for 300 s. To determine rates of plasminogen activation, absorbance measurements were transformed using the first derivative function (dA_{406}/dt). Kinetic constants for the hydrolysis of S-2251 by plasmin ($K_m = 180\ \mu$M, $k_{cat} = 12\ s^{-1}$) were used to convert data into plots of active plasmin concentration against time. In control experiments, we analysed S-2251 hydrolysis in the presence of BT20 cell-conditioned medium and plasminogen (no sct-PA) or in the presence of BT20 cell-conditioned medium and sct-PA (no plasminogen). In both cases, significant substrate hydrolysis was not observed within 600 s. Finally, unconditioned cell culture medium, at the dilution used in our experiments, did not significantly affect the kinetics of activation of [Glu^1]plasminogen or [Lys^{78}]plasminogen by sct-PA.

CK 8 purification

CK 8 was purified by the method of Achtstaetter et al. [21]. Rat hepatocytes were collected as previously described [12] and homogenized in EBSS with 10 mM Hepes, 0.1 mM dithiothreitol (DTT), 1.0 mM PMSF, 2.0 μM E-64, 10 mM EDTA and 10 μM aprotinin. The homogenate was centrifuged at 1250 *g* for 15 min. The resulting pellet was extracted with 1.0% Triton X-100 for 1 h at 4 °C and then subjected to centrifugation at 2500 *g* for 5 min. The pellet was resuspended in 1.5 M KCl, 0.5% Triton X-100, 10 mM Hepes, 5 mM EDTA, 5 mM DTT at 4 °C and forced through a syringe to shear DNA. Centrifugation was performed again. The pellet was rinsed with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, and solubilized in 9.5 M urea, 10 mM Tris/HCl, 5 mM DTT, pH 8.8, for 2 h. This solution, which includes CK 8, CK 18 and some contaminating proteins, was clarified by centrifugation at 14000 *g* for 20 min and dialysed against 8 M urea, 30 mM Tris/HCl, 5 mM DTT, pH 8.8. The preparation was then subjected to DEAE-Sephacel ion-exchange chromatography. The column was equilibrated in 8 M urea, 30 mM Tris/HCl, pH 8.0, and eluted with a linear gradient of 0 to 100 mM guanidine hydrochloride. Samples were screened by absorbance at 280 nm and by SDS/PAGE/Western blot analysis using antibody PCK-26. Fractions containing purified CK 8 were pooled and stored at room temperature.

Ligand blotting

BT20 cell-conditioned medium or purified CK 8 was subjected to SDS/PAGE [22] and electrotransferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) using a Hoefer Transphor apparatus (2 h, 0.5 A). In some studies, the transferred proteins were stained with 0.2% (w/v) Coomassie Blue R250 (Bio-Rad, CA, U.S.A.). Otherwise, the membranes were blocked with 5% non-fat dried milk, rinsed twice with 20 mM sodium phosphate, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.4 (PBS-T), and then incubated with ^{125}I-plasminogen (10 nM) and aprotinin (10 μM), in the presence and absence of 10 mM ϵACA or a 50-fold molar

excess of non-radiolabelled plasminogen. To determine radioligand binding, the blots were rinsed three times for 15 min in PBS-T, dried and autoradiographed, or analysed with a Phosphorimager.

Binding of plasminogen and t-PA to immobilized CK 8

Purified CK 8 (100 μg/ml) in 8 M urea was diluted 1:3 with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, in 48-well cell culture plates and incubated for 1.5 h at 37 °C. The plates were rinsed three times and then blocked by the addition of BSA (10 mg/ml) in EBSS, 10 mM Hepes, pH 7.4, (EHB) for 1.5 h at 37 °C. Control plates were blocked with BSA without prior adsorption of CK 8. All plates were stored at 4 °C until use. The amount of CK 8 immobilized per well was 0.43 ± 0.07 μg ($n = 6$), as determined by BCA assay of SDS-extracted samples.

Plasminogen and sct-PA were radioiodinated using Iodobeads (Pierce). The specific activities of both proteins ranged from 1–2 μCi/μg. Increasing concentrations of either ^{125}I-plasminogen or ^{125}I-sct-PA were incubated in CK 8-coated and control wells for 1 h at 22 °C. Plates were then washed three times with EHB. Bound radioligand was recovered by incubation with 1% SDS, 0.1 M NaOH and quantified in a gamma counter. Non-specific ^{125}I-plasminogen binding was defined as residual binding in the presence of 10 mM ϵACA or a 50-fold molar excess of plasminogen (either method yielded equivalent results). For ^{125}I-sct-PA concentrations up to 0.25 μM, non-specific binding was determined using a 50-fold molar excess of non-radiolabelled t-PA. Non-specific binding of ^{125}I-sct-PA was plotted as a function of ^{125}I-sct-PA concentration and extrapolated to higher concentrations, assuming a continuously linear relationship, in order to conserve material.

Activation of plasminogen bound to immobilized CK 8 as determined by fluorescent substrate hydrolysis

Various concentrations of [Glu^1]plasminogen were incubated in CK 8-coated wells for 1 h at 22 °C. The plates were rinsed three times with EHB to remove unbound plasminogen. sct-PA (10 nM) and the fluorescent plasmin substrate, VLK-AMC (0.5 mM), were then added. Initial rates of substrate hydrolysis were detected by measuring fluorescence emission at 5 s intervals using a Cytofluor 2350 fluorescence plate reader (Millipore). The excitation wavelength was 380 nm and the emission wavelength was 480 nm (5 nm slit widths). Levels of CK 8-associated plasminogen, available for activation by sct-PA, were determined in separate ^{125}I-plasminogen binding studies. Equivalent amounts of plasminogen, in solution, were activated by 10 nM sct-PA in BSA-blocked wells (no CK 8).

In control experiments, hydrolysis of VLK-AMC (0.5 mM) by equal amounts of free (pre-activated) plasmin and CK 8-associated plasmin were compared. The velocity of VLK-AMC hydrolysis was 97 ± 6% higher for free plasmin ($n = 6$).

Activation of plasminogen bound to immobilized CK 8 as determined by SDS/PAGE

^{125}I-[Glu^1]plasminogen (1.0 μM) was incubated in wells with immobilized CK 8 for 1 h at 22 °C. Aprotinin (10 μM) was included in the incubation mixture. After washing the plates three times with EHB to remove unbound plasminogen, 10 nM sct-PA and 10 μM aprotinin were added. At various times, the sct-PA was inhibited by the addition of 10 μM PPACK. Laemmli SDS sample buffer with reductant (7 mg/ml DTT) was then added to the wells. Samples were subjected to SDS/PAGE and autoradiography. Plasminogen activation was detected by conversion of the single-chain zymogen (90 kDa) into the two-chain active species.

When 1.0 μM ^{125}I-[Glu^1]plasminogen was incubated in the CK 8-coated wells, 2.0 ± 0.3 pmol ($n = 5$) associated with the immobilized phase. In control experiments, the equivalent amount of ^{125}I-plasminogen was added to BSA-blocked wells, without CK 8. sct-PA and aprotinin were then added and plasminogen activation was studied, as a function of time, by SDS/PAGE and autoradiography.

RESULTS

Identification of CK 8 in BT20 cell-conditioned medium

BT20 cell-conditioned medium was concentrated 10-fold and subjected to SDS/PAGE and Western blot analysis with antibody PCK-26. A prominent species with an apparent mass of 56 kDa was detected, together with a second less-intense band with an apparent mass of 53 kDa (Figure 1, lane B). The molecular mass of human CK 8 is about 55 kDa [13]. The Western blot analysis suggests that the BT20 cell-conditioned medium contains intact CK 8 (apparent mass of 56 kDa) and a partially degraded form of CK 8. Analysis of three separate preparations demonstrated the same two bands; the amount of the 53 kDa species was slightly increased in one of the three (results not shown).

Plasminogen-binding proteins in the BT20 cell-conditioned medium were identified by ligand blotting. A single protein, with identical electrophoretic mobility to intact CK 8, bound ^{125}I-plasminogen (Figure 1, lane C). No other plasminogen-binding proteins were detected. The 53 kDa band did not bind ^{125}I-plasminogen. This result suggests that the 53 kDa species may be CK 8, proteolytically modified at the C-terminus, so that the C-terminal Lys residue is deleted.

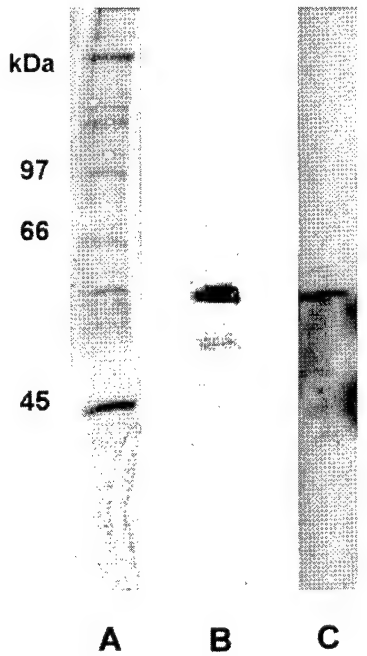


Figure 1 SDS/PAGE analysis of BT20 cell-conditioned medium

Concentrated BT20 cell-conditioned medium (100 μg of protein) was subjected to SDS/PAGE on 10% acrylamide slabs and transferred to poly(vinylidene difluoride) membranes. Resolved proteins were stained with Coomassie Blue (**A**); subjected to Western blot analysis with monoclonal antibody PCK-26 (**B**); or ligand blotted with 10 nM ^{125}I-plasminogen (**C**).

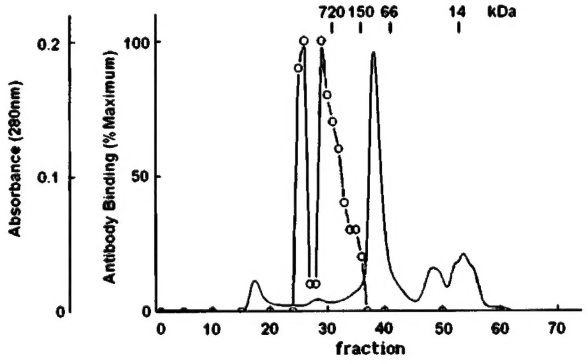


Figure 2 Superose 12 chromatography of BT20 cell-conditioned medium

BT20 cell-conditioned medium (500 μg of protein) was subjected to molecular exclusion chromatography on a Superose 12 column. Protein elution was detected by continuous monitoring of absorbance at 280 nm (unbroken curve). CK 8 was detected by dot–blot analysis with antibody PCK-26 (○).

Characterization of CK 8 in BT20 cell-conditioned medium

BT20 cell-conditioned medium was subjected to molecular exclusion chromatography on a Superose 12 column. CK 8 was detected in elution fractions by dot–blot analysis with antibody PCK-26. Figure 2 shows that the CK 8 was recovered over a wide range of fractions which, by comparison with the chromatography standards, correspond in apparent molecular mass from 150 kDa to over 1000 kDa. These results suggest that the CK 8 in BT20 cell-conditioned culture medium is in the form of variably sized polymers or complexes. The chromatography standards were globular proteins; if the CK-8-containing complexes were highly asymmetric, then the apparent masses are overestimated by comparison with the standards.

Effect of soluble CK 8 on plasminogen activation by sct-PA

Some plasminogen-binding proteins enhance the rate of plasminogen activation by t-PA, especially when the proteins bind plasminogen activator as well [2]. Thus, we tested the ability of BT20 cell-conditioned medium to promote plasminogen activation by sct-PA. It was assumed that any changes in the kinetics of plasminogen activation would be due to CK 8, since CK 8 was the only plasminogen-binding protein in BT20 cell-conditioned medium. Figure 3 shows a representative experiment in which [Glu^1]plasminogen or [Lys^{78}]plasminogen was activated by sct-PA in the presence of S-2251. Initial rates of activation were determined and plotted against plasminogen concentration. In the absence of BT20 cell-conditioned medium, [Lys^{78}]plasminogen was activated more rapidly than [Glu^1]plasminogen, as expected. Removal of the 77-amino-acid N-terminal pre-activation peptide, in [Lys^{78}]plasminogen, causes the zymogen to adopt a more open, readily activated conformation [23,24]. In the presence of conditioned medium, both plasminogens were activated at increased rates. When the BT20 cell-conditioned medium was passed through a sterile Acrodisc 0.2 μm filter (Gelman), the ability of the preparation to promote plasminogen activation was unchanged.

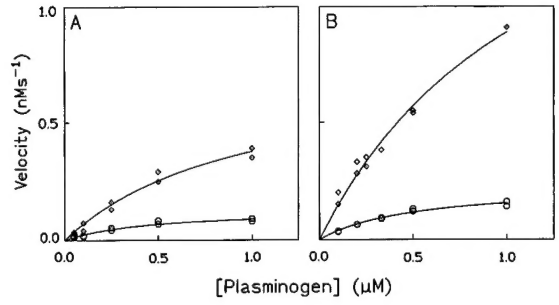


Figure 3 Effects of BT20 cell-conditioned medium on the activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen by sct-PA

Increasing concentrations of [Glu^1]plasminogen (**A**) or [Lys^{78}]plasminogen (**B**) (0.05–1.0 μM) were activated with 2 nM sct-PA in the presence (◇) and absence (○) of a 1:2 dilution of BT20 cell-conditioned medium. The initial velocity of plasminogen activation is plotted against plasminogen concentration.

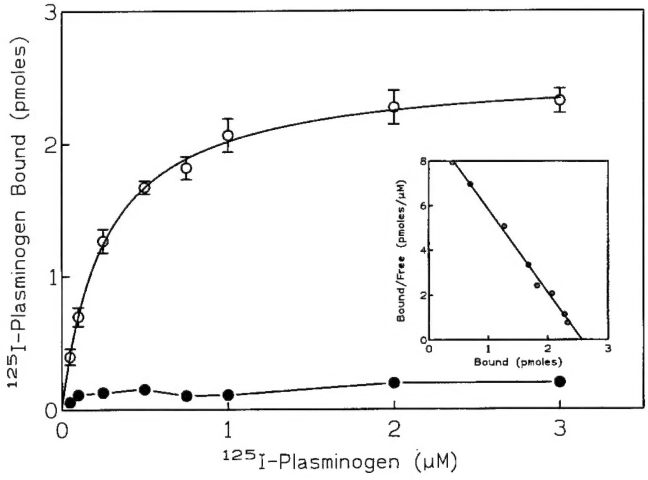


Figure 4 ^{125}I-plasminogen binding to immobilized CK 8

A representative study showing specific binding of ^{125}I-plasminogen to immobilized CK 8 (○) and to BSA-blocked wells (●). Each point represents the average of four replicate determinations (±S.E.M.) in one of four separate experiments. The Scatchard transformation of the data for plasminogen binding to CK 8 is shown in the inset.

The increased rate of activation of [Lys^{78}]plasminogen by sct-PA, in the presence of BT20 cell-conditioned medium, suggests that the mechanism is not limited to plasminogen conformational change. To better address the mechanism by which CK 8 stimulates plasminogen activation, CK 8 was purified from hepatocytes. The effects of purified CK 8 on plasminogen activation were then studied as a model of interactions that may occur with tumour cell-released CK 8.

In separate experiments, the activation of [Glu^1]plasminogen (1.0 μM) by 5 nM tcu-PA was assessed in the presence and absence of BT20 cell-conditioned medium. The [Glu^1]plasminogen was pre-incubated with the conditioned medium for 30 min, according to the standard protocol followed with sct-PA; however, the velocity of plasminogen activation by tcu-PA was not significantly affected by the conditioned medium (the rate of plasminogen activation was decreased by 3 ± 10 % in the presence of the medium, $n = 4$).

Binding of plasminogen and sct-PA to immobilized CK 8

^{125}I-[Glu^1]plasminogen bound to immobilized CK 8 at 22 °C. Figure 4 shows that binding was specific and saturable. By

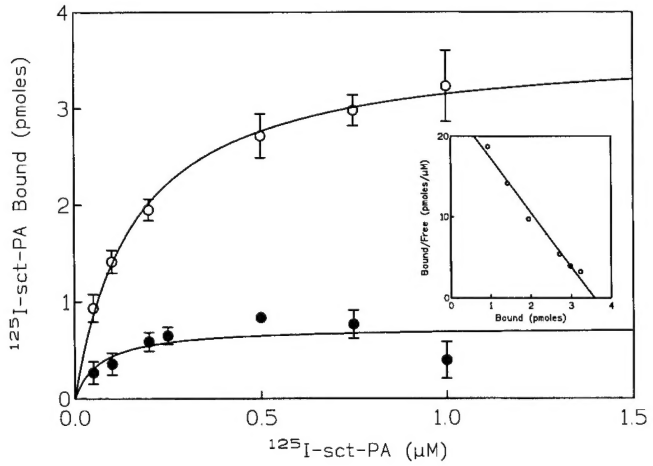


Figure 5 ^{125}I-sct-PA binding to immobilized CK 8

A representative study showing specific binding of ^{125}I-sct-PA to immobilized CK 8 (○) and BSA-blocked wells (●). Each point represents the average of four replicate determinations in one of three separate experiments. The Scatchard transformation of the data for sct-PA binding to CK 8 is shown in the inset.

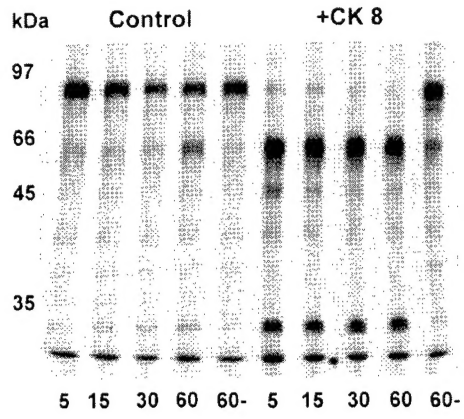


Figure 7 SDS/PAGE analysis of the activation of CK 8-associated ^{125}I-plasminogen by sct-PA

^{125}I-plasminogen, in association with immobilized CK 8 (+CK 8) or free in solution (Control), was activated with 10 nM sct-PA at 22 °C. Reactions were terminated by the addition of 10 μM PPACK at the times (in min) indicated at the bottom of each lane. The contents of each well were recovered in SDS and subjected to SDS/PAGE on 8% gels. Radioactive gels were imaged with a Phosphorimager. Incubations conducted for 60 min in the absence of sct-PA are labelled 60—. Aprotinin (10 μM) was present in each well through the entire incubation.

Scatchard analysis, the K_D was 160±40 nM and the B_{max} was 3.6±0.7 pmol/well ($n = 4$). At saturation, 0.5 mol of plasminogen was bound per mol of immobilized CK 8 monomer. Non-specific plasminogen binding accounted for less than 15% of total binding through the entire plasminogen concentration range. Plasminogen binding to BSA-coated wells was negligible.

^{125}I-sct-PA also bound to immobilized CK 8 at 22 °C (Figure 5). Binding was specific and saturable with a K_D of 250±48 nM, and a B_{max} of 4.0±0.7 pmol/well ($n = 3$). Non-specific binding accounted for less than 10% of total binding for ^{125}I-sct-PA concentrations up to 0.25 μM.

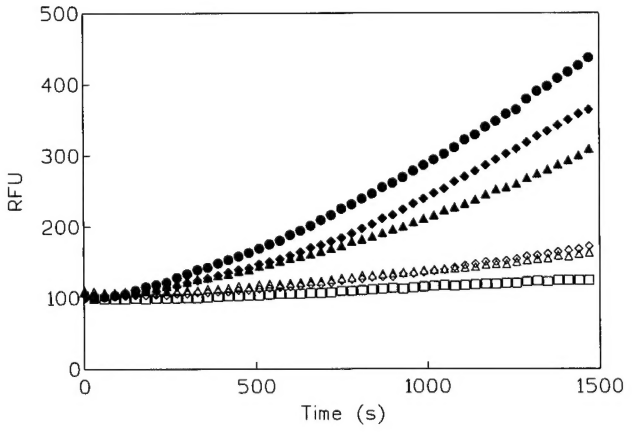


Figure 6 Activation of CK 8-associated plasminogen by sct-PA as determined by fluorescent substrate hydrolysis

CK 8-associated plasminogen [2.0 pmol (●), 1.3 pmol (◆), 1.0 pmol (▲)] was activated with 10 nM sct-PA in the presence of 0.5 mM VLK-AMC. The identical protocol was followed using plasminogen in solution [2.0 pmol (◇), 1.3 pmol (△), 1.0 pmol (□)], in BSA-coated wells. Fluorescent substrate hydrolysis was monitored continuously at 480 nm.

Activation of immobilized CK 8-associated plasminogen as determined by fluorescent substrate hydrolysis

The effects of CK 8 on plasminogen activation were characterized using immobilized CK 8 and the fluorescent plasmin substrate, VLK-AMC. In these experiments, various concentrations of plasminogen were incubated with immobilized CK 8 for 1 h at 22 °C. The wells were washed and activation was initiated by the addition of sct-PA (10 nM) and VLK-AMC (5 mM). The amount of plasminogen bound to the CK 8 had been determined in previous radioligand binding experiments. Thus, we could compare the rates of activation of identical amounts of CK 8-associated plasminogen and plasminogen in solution, in BSA-coated wells. Figure 6 shows that plasminogen activation by sct-PA was accelerated when the plasminogen was CK 8-associated. For the three concentrations of plasminogen studied, the velocity of VLK-AMC hydrolysis was 5–10 times greater for plasminogen that was initially CK 8-associated, compared with plasminogen in solution. Since plasmin that is CK 8-associated hydrolyses VLK-AMC at a decreased rate, the rate of plasminogen activation may have been as much as 20 times greater in the CK 8-containing wells.

SDS/PAGE analysis of ^{125}I-plasminogen activation by sct-PA

In continuous assays (activator, plasminogen and plasmin substrate present in the same solution), plasmin substrates can affect the rate of plasminogen activation [25,26]. Thus, SDS/PAGE experiments were performed to confirm that CK 8 accelerates the rate of [Glu^1]plasminogen activation by sct-PA. ^{125}I-[Glu^1]plasminogen (1 μM) was incubated with immobilized CK 8 for 1 h at 22 °C. After washing the wells, 10 nM sct-PA and 10 μM aprotinin were added. At various times, the sct-PA inhibitor, PPACK (10 μM), was added to stop the reaction. The contents of the wells were solubilized with SDS and subjected to SDS/PAGE. ^{125}I-[Glu^1]plasminogen activation was detected as conversion of the single-chain form of the protein into the two-chain form (apparent masses of 60 and 35 kDa). Figure 7 shows that the majority of the CK 8-associated ^{125}I-[Glu^1]plasminogen

was converted into plasmin within 5 min. By contrast, the identical amount of ^{125}I-[Glu^1]plasminogen remained primarily in the zymogen form when incubated with 10 nM sct-PA in BSA-coated wells. No activation was observed when CK 8-associated plasminogen was incubated for 1 h at 22 °C without sct-PA (Figure 7, lane 60−).

Equivalent experiments were performed with [Lys^{78}]plasminogen and sct-PA. In the absence of CK 8, the [Lys^{78}]plasminogen was activated at a more rapid rate compared with [Glu^1]plasminogen, as expected. When the [Lys^{78}]plasminogen was initially CK 8-associated, the rate of activation was substantially increased. Almost all of the plasminogen was converted into the two-chain form in 5 min (results not shown). These studies and the experiments with BT20 cell-conditioned medium demonstrate that the effects of CK 8 on plasminogen activation are not restricted to the closed plasminogen conformation adopted selectively by [Glu^1]plasminogen.

DISCUSSION

Plasmin activity is controlled locally by the action of plasminogen activators, such as t-PA and u-PA, and by plasmin inhibitors, including α_2-antiplasmin and α_2-macroglobulin [1,2]. A second level of control is imposed by proteins which bind plasminogen via kringle domain lysine-binding sites. These proteins, which include soluble factors, extracellular matrix components and plasma membrane receptors, alter the rate of plasminogen activation by t-PA and u-PA [1,2,27,28]. The same factors also decrease the reactivity of bound plasmin with inhibitors. Thus, kringle domain-dependent interactions profoundly influence the concentration of available plasmin in various micro-environments.

In the present study, we demonstrated the presence of soluble CK 8-containing complexes in the conditioned medium of breast carcinoma cells, confirming previous studies [18]. The soluble CK 8 bound plasminogen and enhanced the rate of plasminogen activation by t-PA. In experiments with purified immobilized CK 8, we demonstrated that CK 8 has the capacity to bind both t-PA and plasminogen. Thus, CK 8 may promote plasminogen activation on the cell surface and in the pericellular spaces surrounding breast cancer cells, *in vivo*.

Plasminogen exists in at least two conformations that are differentially activated [1,23,24]. In the presence of physiological concentrations of Cl^-, [Glu^1]plasminogen exists primarily in the 'closed' conformation which is relatively resistant to activation [29]. [Glu^1]Plasminogen is converted into the more readily activated 'open' conformation by plasmin-mediated cleavage of the N-terminal peptide to yield [Lys^{78}]plasminogen or by binding ω-amino acids such as ϵACA [2,24,29]. Proteins that bind to the kringle domains may also convert [Glu^1]plasminogen into the open conformation and thereby promote [Glu^1]plasminogen activation [30]. By contrast, the conformation of [Lys^{78}]plasminogen is minimally affected by lysine-binding interactions [1,24]. In our experiments, with BT20 cell-conditioned medium and purified CK 8, an increased rate of plasminogen activation was observed with both [Glu^1]- and [Lys^{78}]plasminogen, suggesting that plasminogen conformational change is not exclusively responsible for the increased rate of activation. An alternative model is that which has been proposed for fibrin [3]. t-PA and plasminogen bind to fibrin, forming a ternary complex in which the plasminogen is activated more readily. We hypothesize that a similar ternary complex forms with CK 8; however, based on the studies completed to date, we cannot rule out the possibility that CK 8 functions primarily as an enzyme (t-PA) modulator without requiring plasminogen binding for enhanced activation.

Recently, we identified CK 8 as a candidate plasminogen receptor on several epithelial and carcinoma cell lines [12]. While most of the cellular CK 8 is contained within highly insoluble filaments, CK 8 may also exist as a component of soluble complexes intracellularly and in the extracellular spaces [15,18,19]. Intracellular soluble cytokeratin is in the form of heteropolymers; little or no cytokeratin exists as complexes smaller than a tetramer [31]. Bachant and Klymkowsky [32] subjected soluble cytokeratins from *Xenopus* oocytes to molecular exclusion chromatography and found that the majority of the protein eluted with an apparent mass of 750 kDa, while a smaller amount eluted at 150 kDa. Our chromatography analysis of CK 8 released by tumour cells showed similarly sized complexes, although some of the CK 8 also eluted in the void volume. At the present time, we do not understand the relationship of released CK 8 to soluble and insoluble forms of intracellular cytokeratin; however, the CK 8-containing complexes released from the BT20 cells are probably equivalent to Tissue Polypeptide Antigen. The ability of tumour-secreted, soluble CK 8 to enhance plasminogen activation suggests that Tissue Polypeptide Antigen may influence fibrinolysis *in vivo*.

Expression of CK 8 is usually restricted to simple epithelia; however, numerous studies have shown that CK 8 is aberrantly expressed in malignant cells [33–35]. Aberrant expression of CK 8 may correlate with an invasive phenotype. In transitional cell carcinoma and squamous cell carcinoma, aberrant expression of CK 8 is localized to the tumour invasion front [36,37]. In malignant melanoma, aberrant expression of CK 8 is correlated with an increase in tumour invasiveness, *in vitro* [33]. Most significantly, mouse L fibroblasts, which lack CK 8, show increased motility and penetration of Matrigel after transfection with CK 8/CK 18 [38]. Motility and cellular invasion through Matrigel are processes that are promoted by cell-surface-associated plasmin. The studies presented here suggest a model that may partially explain the correlation of CK 8 expression with cellular invasiveness. We propose that the small fraction of total cellular CK 8, which is expressed on the outer cell surface, may promote cellular invasiveness by enhancing proteinase activation in the pericellular spaces.

This work was supported by grant DAMD 17-94-J-4450 from the Department of the Army (USA) Breast Cancer Research Program.

REFERENCES

1 Ponting, C. P., Marchall, J. M. and Cederholm-Williams, S. A. (1992) Blood Coagulation Fibrinolysis **3**, 605–614
2 Henkin, J., Marcotte, P. and Yang, H. (1991) Prog. Cardiovasc. Dis. **34**, 135–164
3 Hoylaerts, M., Rijken, D. C., Lijnen, H. R. and Collen, D. (1982) J. Biol. Chem. **257**, 2912–2919
4 Plow, E. F., Herren, T., Redlitz, A., Miles, L. A. and Hoover-Plow, J. L. (1995) FASEB J. **9**, 939–945
5 Hajjar, K. A., Harpel, P., Jaffe, E. and Nachman, R. L. (1986) J. Biol. Chem. **261**, 11656–11662
6 Gonias, S. L., Braud, L. L., Geary, W. A. and VandenBerg, S. R. (1989) Blood **74**, 729–736
7 Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. and Magnusson, S. (1978) Prog. Chem. Fibrinolysis Thrombolysis **3**, 191–209
8 Christensen, U. (1985) FEBS Lett. **182**, 43–46
9 Fleury, V. and Angles-Cano, E. (1991) Biochemistry **30**, 7630–7638
10 Miles, L. A., Dahlberg, C. M., Piescia, J., Felez, J., Kato, K. and Plow, E. F. (1991) Biochemistry **30**, 1682–1691
11 Hajjar, K. A., Jacovina, A. T. and Chacko, J. (1994) J. Biol. Chem. **269**, 21191–21197
12 Hembrough, T. A., Vasudevan, J., Allietta, M. M., Glass, W. F. and Gonias, S. L. (1995) J. Cell Sci. **108**, 1071–1082

13 Moll, R., Franke, W. W. and Schiller, D. L. (1982) Cell **31**, 11–24
14 Morita, T., Tondella, M. L., Takemoto, Y., Hashido, K., Ichinose, Y., Nozaki, M. and Matsushiro, A. (1988) Gene **68**, 109–117
15 Leube, R. F., Bosch, F. X., Romano, V., Zimblemann, R., Hofler, H. and Franke, W. W. (1986) Differentiation **33**, 69–85
16 Weber, K., Osborn, M., Moll, R., Wiklund, B. and Luning, B. (1984) EMBO J. **3**, 2707–2714
17 Bjorklund, B. and Bjorklund, V. (1983) Cancer Detect. Prevent. **6**, 41–50
18 Chan, R., Rossitto, P. V., Edwards, B. F. and Cardiff, R. D. (1986) Cancer Res. **46**, 6353–6359
19 Brabon, A. C., Williams, J. F. and Cardiff, R. D. (1984) Cancer Res. **44**, 2704–2710
20 Deutsch, D. G. and Mertz, E. T. (1970) Science **170**, 1095–1096
21 Achtstaetter, T., Hatzfeld, M., Quinlan, R. A., Parmelee, D. C. and Franke, W. W. (1986) Methods Enzymol. **134**, 355–371
22 Laemmli, U. K. (1970) Nature (London) **227**, 199–205
23 Mangel, W., Lin, B. and Ramakrishnan, V. (1990) Science **248**, 69–73
24 Violand, B. N., Byrne, R. and Castellino, F. J. (1978) J. Biol. Chem. **253**, 5395–5401
25 Kolev, K., Owen, W. G. and Machovich, R. (1995) Biochim. Biophys. Acta **1247**, 239–245
26 Machovich, R. and Owen, W. G. (1993) Thromb. Haemostasis **70**, 864–866
27 Knudsen, B. S., Silverstein, R. L., Leung, L. L., Harpel, P. C. and Nachman, R. L. (1986) J. Biol. Chem. **261**, 10765–10771
28 Stack, S., Gonzalez-Gronow, M. and Pizzo, S. V. (1990) Biochemistry **29**, 4966–4970
29 Urano, T., Chibber, B. and Castellino, F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4031–4034
30 Pannell, R. and Gurewich, V. (1986) Blood **67**, 1215–1223
31 Fuchs, E. and Weber, K. (1994) Annu. Rev. Biochem. **63**, 345–382
32 Bachant, J. B. and Klymkowsky, M. W. (1996) J. Cell Biol. **132**, 153–165
33 Hendrix, M. J. C., Seftor, E. A., Chu, Y.-W., Seftor, R. E. B., Nagle, R. B., McDaniel, K. M., Leong, S. P. L., Yohem, K. H., Leibovitz, A. M., Meyskens, F. L., Conaway, D. H., Welch, D. R., Liotta, L. A. and Stetler-Stevenson, W. (1993) J. Natl. Cancer Inst. **84**, 165–174
34 Knapp, A. C., Bosch, F. X., Hergt, M., Kuhn, C., Winter-Simanowski, S., Schmid, E., Regauer, S., Bartek, J. and Franke, W. W. (1989) Differentiation **42**, 81–102
35 Franke, F. E., Schachenmayr, W., Osborn, M. and Altmannsbergen, M. (1991) Am. J. Pathol. **139**, 67–79
36 Schaafsma, H. E., Ramaekers, F. C. S., van Muijen, G. N. P., Robben, H., Lane, E. B., Leigh, I. M., Ooms, E. C. M., Schalken, J. A., van Moorselaar, R. J. A. and Ruiter, D. J. (1991) Am. J. Pathol. **139**, 1389–1400
37 Schaafsma, H. E., van der Velden, L. A., Manni, J. J., Peters, H., Link, M., Ruiter, D. J. and Ramaekers, F. C. S. (1993) J. Pathol. **170**, 77–86
38 Chu, Y.-W., Runyan, R. B., Oshima, R. G. and Hendrix, M. J. C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 4261–4265

Received 2 November 1995/1 March 1996; accepted 25 March 1996